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**STUDIES ON THE DEVELOPMENT AND ULTRASTRUCTURE
OF PITS IN CONIFEROUS XYLEM**

YUJI IMAMURA

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Introduction

It is well known that both the physical and chemical properties of wood are closely related to the wood structure. The permeability of wood is mainly dependent on its porous structure and it has been understood that liquids in the secondary xylem of softwoods mostly move through the pore of the cell, the pits.

The structure of pits has received attention for a long time in plant anatomy, and since electron microscope was used for the elucidation of wood ultrastructure, the pit was one of the first and most interested object to be studied. Even now new facts about their fine structure are being published.

It is generally observed that the softwood tracheids have "bordered pit". Pairs of the bordered pits are separated by a membrane with a thickened center, called "torus", and radially oriented fibrils, "margo". By contrast, the pits in the parenchyma cells do not have the overarching border and are termed "simple pit".

Though ultrastructure of the pit has been studied earnestly, many problems are still remained unsolved. Elucidation of the development of pits is one of the subjects which should be examined in relation to the cell wall formation and organization. When the development and ultrastructure of the pits are investigated, there are some difficulties due to the complexity of the cell wall. Then, a more suitable method examining the cell wall structure is required to explicate these problems.

In the present investigation, the development and ultrastructure

of pits of tracheids and parenchyma cells are studied using new technique of observation of the inner surface of the differentiating cell.

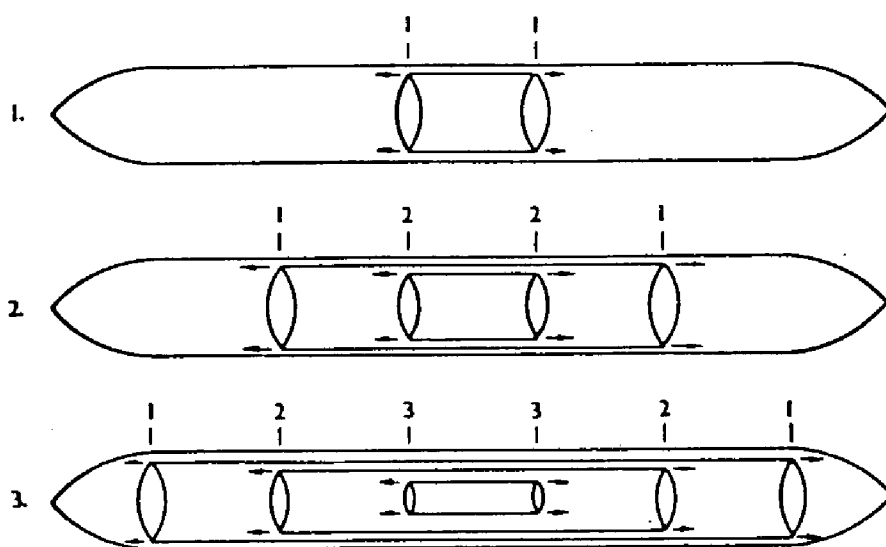
1. Electronmicroscopy of the inner surface of cell wall in differentiating xylem

The study on the wood ultrastructure has been advanced by the progress of electron microscopy. However, the cell wall organization is so complicate that the more suitable method for examination of the cell wall structure has been expected (Dunning 1969a).

It is assumed that the inner surface of the tracheids in a differentiating radial file shows a sequential development of the cell wall formation. The cell wall is formed by successive deposition of the primary wall (P.W.) and the secondary wall (S. W.), subsequent to the cell division in the cambial zone. The examination of the inner surface of the differentiating tracheids by the replication method may allow observation of the cell wall layers in the different formation stages.

Wardrop and Harada (1965) proposed by optical and autoradiographic observations of cells from *Eucalyptus regnans* and *Pinus radiata* grown in an atmosphere of labelled carbon dioxide that the secondary wall formation begins near middle of the cell and proceeds toward the ends (Text-Fig. 1). This hypothesis suggests that one could observe the progress deposition of several lamellae having the different microfibrillar orientations in the inner surface of one tracheid.

There are some difficulties to observe the inner surface of the differentiating cell for replication. One of them is that the cytoplasm in the differentiating cell is attached to the surface of the wall, resulting in obscuring the cell wall organization. Another



Text-Fig. 1 Diagrammatic representation of the formation of three successive lamellae of the outer layer of the secondary wall in a differentiating fiber (Wardrop, Harada 1965).

difficulty is the morphological artifact caused by drying. The cell wall in the developing condition sometimes causes the collapse and curling through drying. To overcome these difficulties, two procedures were employed in the present investigation; the plasmolysis treatment and freeze-drying technique.

Materials

Pinus densiflora Sieb. et Zucc.

Cryptomeria japonica D. Don.

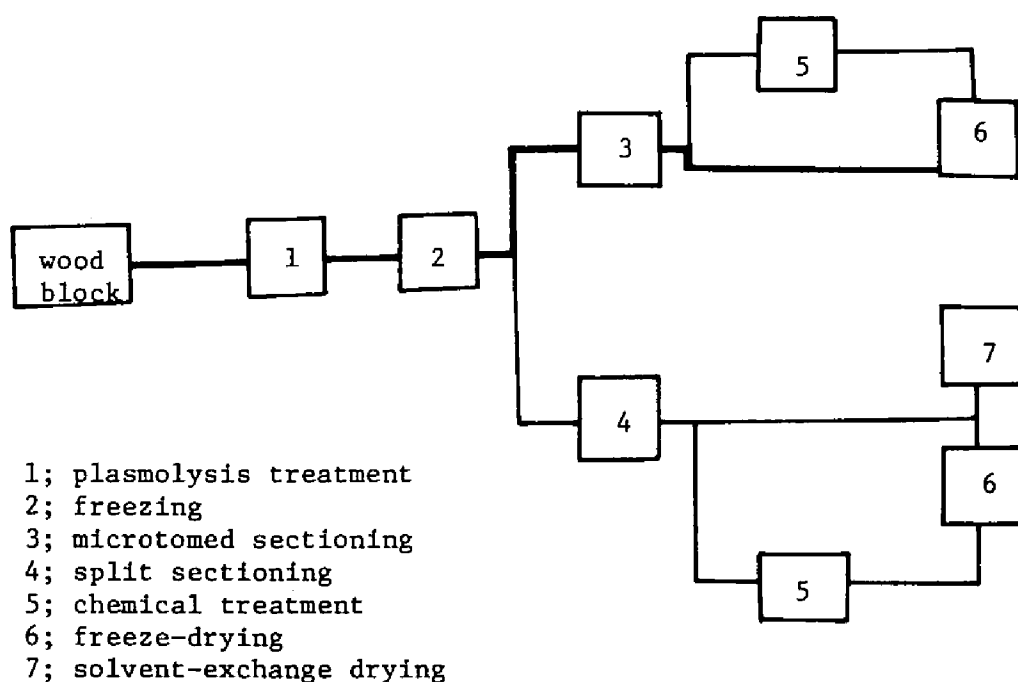
Chamaecyparis obtusa Endl.

Abies firma Sieb. et Zucc.

Each tree was harvested in Kamigamo Experimental Forest Station of Kyoto University Forest. Small wood blocks (about 10mm x 10mm x 10mm) containing the differentiating xylem were collected from the stem of each tree throughout the growing season (April - July).

Preparation of sample for replication

The key to elucidate the problem of the present study was to prepare the section showing the inner surface of the differentiating cell for replication of electron microscopy. Following methods were developed to satisfy the purpose (Text-Fig. 2).



Text-Fig. 2 A flow chart of the employed methods .

Wood blocks were immediately immersed into a hypertonic solution of a saccharose (0.8 M) and were kept for 2 days at 4° C (Text-Fig. 2-1). By this treatment the cytoplasm was detached from the newly-formed cell wall under plasmolysis. The blocks were then frozen in a dry ice-ethanol bath and stored in frozen (Text-Fig. 2-2).

The present examination using electron microscopy was based mainly on the observation of replicas of the inner surface of the differentiating tracheid wall. The sections were cut with a sliding microtome to expose the inner surface of the tracheid wall and used for replication. Radial longitudinal sections about 30-40 microns in thickness were made using a sliding microtome equipped with a thermoelectric freezing unit (Text-Fig. 2-3). The cytoplasmic substances in the differentiating tracheids were rinsed away in distilled water, and then the sections were again frozen on glass slides and freeze-dried (Text-Fig. 2-6).

Some of the sections were treated with Jeffrey's solution (a mixture of 7.5% chromium trioxide and 7.5% nitric acid, 1:1) at 30°C for 8 hours to improve profiling of cellulose microfibrils of the cell wall by removing the amorphous materials (Text-Fig. 2-5).

For the observation of pit membranes in the stage of the secondary wall formation of tracheids, split radial sections were employed (Text-Fig. 2-4). Some split sections were freeze-dried and others were dried by solvent-exchange drying procedure (Text-Fig. 2-7). The solvent-exchange drying was accomplished by sequentially extracting the specimens with ethanol, acetone, and pentane in a Soxhlet

apparatus following the procedure by Thomas and Nicholas (1966).

These dried sections were stored in a desiccator over silica-gel until replicated.

Replicating procedure

The direct carbon method by Côté et al. (1964) or Tsoumis (1964) was improved to avoid the contamination owing to the backing materials and to pick up the replica films easily.

The steps in replicating procedure are as follows ; 1) Shadowcast with platinum-palladium at an angle of approximately 30°-45° to the horizontal in a bell jar of the evaporation apparatus. 2) Evaporate carbon at an angle of approximately 75°-90° with rotating the specimen. 3) Prepare a polystyrene disk by placing polystyrene granules on a glass slide, heating on a hot plate at 150°C until soft, and then pressing between two slides. 4) Affix the shadowed and carbon-coated sample to the polystyrene disk through heating at about 90°C. 5) Immerse the sample affixed to the polystyrene disk into 72% sulfuric acid and then 10% Jeffrey's solution. 6) Rinse the sample with distilled water and then dry it. 7) Score the replica into about 3 mm squares. 8) Immerse the sample into toluene, and pick up the floating replica film on grids.

Preparation of sections

The ultra-thin sections for electron microscopy were prepared in the conventional manner. For preparing stained sections, the samples were fixed and stained with KMnO_4 , dehydrated with ethanol series,

embedded in epoxy resin, and cut with a Porter-Blum ultramicrotome. The ultra-thin sections were picked up on grids coated with chloroprene rubber and dried.

To obtain shadowed sections, the sample was dehydrated and embedded in methacrylate in the conventional manner. The sections mounted on grids were immersed in toluene to remove the resin and shadowed with platinum-palladium in a bell jar of the evaporation apparatus.

The replicas and sections were examined in a JEM 7 electron microscope.

1.1. Plasmolysis treatment

The inner surface of a differentiating cell is covered with cell components such as cytoplasm, when the microtomed section is prepared without any plasmolysis treatment (Fig. 1). The adhesion of the cytoplasm to the inner surface of the cell wall is recognized not only in the differentiating tracheid, but also in the ray parenchyma cell in the sapwood, resulting in veiling of the microfibrillar organization.

The application of plasmolysis treatment allowed the observation of the inner surface of the cell wall free of the adhering substances (Figs. 2,3). In sectional view, the wall surface is detached from the cytoplasm, showing the naked surface of the cell wall (Fig. 4).

The wall surface showing clear appearance of the microfibrillar

organization is also recognized in the tracheid in the other developing stage (Fig. 5), and in the ray parenchyma cell (See chapter 3).

The sample which was immediately frozen after sectioning without rinsing in water, shows the inner surface attached with granular materials which can be removed by sufficient rinsing. Too much rinsing may alter the microfibrillar organization of the newly formed wall.

When the cell wall structure is studied through the observation of its development, it is expected that the microfibril orientation in the newly formed surface is not changed in the following differentiation. Wardrop and Harada (1965) observed the replica of the radial sections containing the differentiating xylem. They noted that the different layers of the S_1 , S_2 and S_3 resembled to the texture of these layers of mature cells, so that any changes in microfibril orientation did not occur following their formation.

Preston (1964) proposed the end-growth hypothesis in the formation of microfibrils. In preparing the material for micrography, the cytoplasm in the cells of *Cladophora* and *Chaetomorpha*, was removed by plasmolysis, hence the granules only hint at the nature of the active cytoplasm surface. In the present study, the newly formed wall, the surface appearance of which was similar to that presented by him was obtained (Fig. 7). Recently new findings have been gained by the electron microscopic study on the cell wall-cytoplasm interface of the differentiating cell (Fujita, Saiki 1971).

1.2. Drying methods

When soft wet specimens—pulp, unlignified wood and differentiating xylem—are dried by evaporation in air or under vacuum, they are shrunk and distorted. As the receding surface of evaporating water passes the specimen, surface tension collapses, distorts and often ruptures it.

Principally there are some ways to lead specimens which are in water saturated condition to their dry state without the formation of any artifact. Drying techniques to maintain the original state of wood structure have been invented to observe the bordered pit membrane in unspirated condition.

Through the application of solvent-exchange drying techniques, Thomas and Nicholas (1966) were able to depict bordered pit membranes in unspirated state. The solvent-exchange drying was accomplished by sequential extraction of the specimens for 24 hrs. with each of methanol, acetone and pentane in a Soxhlet apparatus. After removal from pentane, the specimens were dried at 65°C for 15 minutes.

For the critical-point drying method as described by Anderson (1951), specimens were impregnated with methanol, amylacetate and finally with liquid carbon dioxide. At this point, the temperature was raised above the critical point (34°C). Specimens dried in this manner are not subjected to liquid surface tension force.

In the freeze-dry method the specimen is frozen as rapidly as possible, kept cold, and dried under continuous vacuum.

Thomas (1969) indicated that none of the solvent-exchange, critical-point and freeze-drying caused pit aspiration and showed any

difference in the appearance of the pit membrane structure among them.

Although the freeze-drying technique has a disadvantage to need a long time in drying, it is a good method in keeping the original state of the chemical components of the cell wall. The solvents required in other two methods, may remove various incrusting materials as shown by Thomas (1969) and in the present study (Figs. 8, 9).

In the present study dry ice-ethanol solution was used to freeze the samples. Then the samples were transferred into a glass-jar which was cooled previously by immersion in the solution, and kept in vacuum condition for about 2 days. This drying method is good in keeping the original state of wood structure except some distortion. In order to obtain the best structural condition, the wet specimen must be cooled as fast as possible and kept in very low temperature (Nei 1970).

It is known whether the figures obtained through the method give absolutely the same pictures to those of the water saturated surface, although the pictures are assumed to reflect the original state.

1.3. Summary

A preparation method for electron microscopic studies through replication of the inner surface of cell wall in the differentiating cell is described. Two treatments are performed in the preparation of the material for replication: 1. the removal of cytoplasm by plasmolysis and 2. the application of a freeze-drying method to keep the newly formed wall in native condition. Through application of this method to the differentiating xylem, it is possible to detect

the innermost surface of the cell wall which is detached from cytoplasm and free of the artificial modifications induced by drying. This method makes it useful to observe the organization of wood cell wall in the process of its formation.

2. Development and ultrastructure of the bordered pits of tracheids

2.1. Development of the bordered pit membrane

The ultrastructure of bordered pits in coniferous tracheids, as a modified region of the cell wall organization or as a main route in the movement of liquids within the wood, has attracted considerable attention from many investigators.

The observations of bordered pit formation have been performed using mainly *Pinus* tracheids. As a result of the electron microscopic study of the bordered pit membrane from macerated tracheids of Scots pine (*Pinus sylvestris* L.), Frey-Wyssling et al. (1956) stated that at first the pit membrane possessed a typical primary wall organization, and later the bundles of radially oriented microfibrils suspending the torus were formed by rearranging the existing microfibrils of the primary wall and possibly also by the addition of the newly-formed radial microfibrils on them. On the other hand, Jayme et al. (1960) proposed a new concept regarding a causal relationship between pit aspiration and the presence of a torus. This opinion is that the torus suspended by radially oriented microfibrils is not a normal occurrence but a result of pit aspiration. Fengel (1966) suggested that throughout the differentiating period of the tracheids the margo was heavily encrusted in matrix substances and that at the conclusion of differentiation of the tracheids it appeared to be perforated.

The use of the technique of solvent-exchange drying prevents pit aspiration and allows the observation of the pit membranes which are

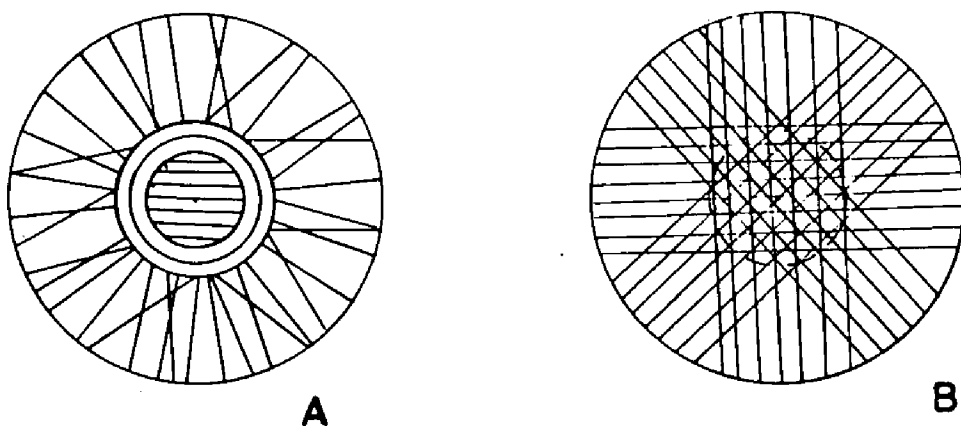
non-aspirated both in mature and in differentiating stages (Thomas 1968). Thomas indicated through the examination of split radial surface that the bordered pit membrane which depicted the torus and radiating microfibrils of the margo were embedded in matrix substances throughout their formation. He also supposed that at first a secondary apposition of large radiating microfibrils occurred in the margo region and then the torus began its formation with the deposition of circularly oriented microfibrils in the central region of the pit membrane followed by apposition of amorphous substances, and that as the pit membranes approached maturity their matrix substances were removed exposing a margo structure of microfibrils.

Bauch et al. (1968) investigated the development and chemical composition of the pit membranes in tracheids of several coniferous species through histological optic microscopy. They assumed that the formation of a pit membrane might already be recognized in the primary wall stage and that in this phase the torus was almost developed while the margo remained embedded in matrix until cell differentiation was completed.

However, the conclusion of the pit membrane formation or the removal of matrix substance from margo has not been studied in relation to the development of the tracheid wall. Also, the arrangement of microfibrils in the pit membrane encrusted with the matrix substances has not yet been demonstrated. The first purpose of the present study is to obtain information on the formation of the bordered pit membrane of *Pinus densiflora*, through the observation of the inner

surface of the differentiating tracheid.

Harada (1964) noted that the bordered pit membrane of coniferous tracheids were of two different types. In the one type they had the circularly oriented microfibrils around the periphery of the torus, and in the other type they showed the continuous passage of microfibrils from margo to torus surface (Text-Fig. 3). The next object of this study was the examination of the development of the bordered pit membranes of *Cryptomeria japonica* and *Chamaecyparis obtusa*, which belong to the latter type of pit membrane noted by Harada.



Text-Fig. 3 Diagrammatic representation of the pit membrane structure of longitudinal tracheid in softwood. A; *Pinus* type bordered pit membrane, B; *Taxus* type bordered pit membrane. (Harada 1964)

The bordered pits between ray tracheid and longitudinal tracheid appear to be effective communication channels for intertracheid flow of liquids in some coniferous woods. It has been also said that the bordered pit membranes between ray-longitudinal tracheids were considerably different in structure from those interconnecting

longitudinal tracheids. The development of the bordered pit membrane between ray-longitudinal tracheids is also presented.

2.1.1. Pinus type bordered pit membrane

It has been established from microscopic studies on the primary wall in the differentiating xylem that the outer surface of the primary wall exhibits a loose network of axially or irregularly oriented microfibrils, whereas the inner surface of the primary wall has transversely oriented microfibrils (Wardrop, Harada 1965). Fig. 10 shows the inner surface of a differentiating cell wall in the very early stage of cell wall formation, which is predicted to develop to a tracheid although the cell has not yet attained its final length and width at this stage. It is demonstrated from Fig. 10 that the so-called primary pit field is bordered by the aggregation of transversely oriented microfibrils and possesses microfibrils encircling the pores which are assumed to be the plasmodesmata. As noted by Kerr and Bailey (1934) and by Roelofsen and Houwink (1953), during the elongation phase of cell growth, the primary wall is stretched, so that the area of a primary pit field enlarges.

When the differentiating tracheid has reached its final length, the radiating microfibrils appear in the vicinity of the central portion of the primary pit field where the torus should develop in the later stage (Fig. 11). The presence of microfibrils with circular orientation around the periphery of the torus can be recognized in the more developed tracheids. When the differentiating tracheid

reaches the final stage in the formation of the outer layer of the primary wall, the bordered pit membranes are almost developed so that the torus may be distinguished from the margo having the radiating microfibrils (Fig. 12). It is also shown that the torus which is thicker than the margo region reveals the circular orientation of the microfibrils and that the margo consists of radiating microfibrils embedded in matrix substances. This evidence reveals that the circularly oriented microfibrils on the torus are not the secondary wall, but the primary wall origin.

Accordingly it is sure that in the phase of the primary wall formation the pit membrane has the same structure as a mature one. However, it is demonstrated from the observation of freeze-dried samples that the margo of the pit membrane is embedded in amorphous materials so that the fine structure of the margo cannot be clearly recognized. Using an agent such as Jeffrey's solution, the amorphous materials are removed from the pit membrane, and the microfibrillar structure of the margo is clearly observed as shown in Fig. 13. The torus and margo which have finished their development are indicated in this figure, showing the identical structure of microfibrils as the mature one.

Fig. 14 illustrates the structure of the non-aspirated pit membrane in the differentiating zone as shown by Thomas (1968) through the solvent-exchange drying, but this sample was prepared using the freeze-drying method. It may be noted that, although the microfibrils of the torus and margo are completely formed, the microfibrils of the

margo are fully embedded in the matrix substances. The application of solvent-exchange drying technique to the sample results in the extraction of some amount of the incrusting material from the pit membrane as well prevention of pit aspiration (Fig. 15). It can be seen in Fig. 15 that some part of the margo in the pit membrane is already perforated.

Fig. 16, which is a micrograph of the embedded pit membrane with amorphous material, reveals the outer surface of the pit border facing the pit chamber, with the warty layer visible through the encrusted margo partially torn away. Although this tracheid is supposed to be fully mature because of the presence of a warty layer on the pit border, the margo is not evenly perforated but still embedded in non-cellulosic substances. However, the more developed tracheid next in radial file to the one shown in Fig. 16 possesses a pit membrane which is completely perforated (Fig. 17).

As described above, the bordered pit membrane has concluded its development of form and structure in the stage of the primary wall formation, and throughout the differentiating zone it has been embedded in the matrix substances. After the warty layer has been formed in the tracheid wall, the removal of the matrix substances in the margo occurs rapidly to make the margo perforated.

2.1.2. Taxus type bordered pit membrane

In the bordered pit membrane of an earlywood tracheid from *Cryptomeria japonica* or *Chamaecyparis obtusa*, the margo microfibrils

run continuously over the torus surface (Fig. 18). This pit membrane is different in the torus structure from that of *Pinus* tracheids. In *Pinus* tracheids the bordered pit membrane was shown to be embedded in amorphous substances throughout the differentiating zone (Thomas 1968, Imamura et al. 1973). The developing bordered pit membrane of *Cryptomeria japonica* or *Chamaecyparis obtusa* was also found to be embedded in amorphous substances, showing a similar appearance to that of *Pinus* tracheid (Fig. 19). Radiating strands of the margo can be observed in the amorphous substances, which are not accompanied by circularly oriented microfibrils in the periphery of the torus. The removal of the amorphous substances by chemical treatment made clear the microfibrillar structure of the torus and margo (Fig. 21). Through the interstices in the margo it can be observed that the pit border of the adjacent tracheid has not fully developed. This micrograph also indicates that the microfibrillar structure of the pit membrane has been fully constructed and that it depicts the same structure as the mature one. Though Figs. 19 and 20 show the split radial surfaces from the differentiating tracheids, Fig. 21 indicates the inner surface of the tracheid in the early stage of its development.

In Fig. 21, it is obvious from the wavy orientation of microfibrils in the unpitted area and from little development of the pit border, that this tracheid is in the later stage of the primary wall formation. The wave-like orientation of microfibrils has been said to be the characteristic of the inner surface of the primary wall (Wardrop, Harada 1965). It is observed through the broad pit aperture that the

microfibrillar structure of the torus and margo has been fully constructed. This fact is coincident with the evidence found in the *Pinus* tracheid. The radiating microfibrils of the margo could be detected even in the earlier stage in which the pit border development had not yet initiated.

From the observations of the present study, full development of microfibrillar structure of the torus and margo in the stage of the primary wall formation and the embedding of the margo throughout the differentiating zone are supposed to be general phenomena which occur in the process of bordered pit membrane formation in coniferous tracheids.

2.1.3. Development of ray-longitudinal tracheid bordered pit membrane

Ray tracheids appear in the wood of some conifers. The development of the bordered pit membrane between ray and longitudinal tracheid was also examined using wood of *Pinus densiflora*. The pit membrane in the differentiating zone was also shown to have a very similar appearance to that interconnecting longitudinal tracheids. It was observed that the microfibrillar structure of the torus and margo had been constructed in the later stage of the primary wall formation. The margo was observed to be embedded in the amorphous substances throughout the differentiating zone (Fig. 22). As the developmental stage proceeded to the maturity, the embedding substances, which were fully removed in the earlywood bordered pit membrane interconnecting longitudinal tracheids, survived sometimes partially in the ray tracheid

bordered pit membrane (Fig. 23).

According to Thomas and Nicholas (1969) the pit membrane between ray and longitudinal tracheid took on two different structures. In the first type the margo microfibrils continued over the torus surface, while in the second type the circular orientation of microfibrils was detected on the torus. In this study, both types of the bordered pit membranes were also recognized. The pit membrane with the torus of the relatively small diameter generally exhibits the first type of the torus structure, and that with the torus of the larger one does the latter type. The dense structure of the margo microfibrils is apt to be observed in the pit membrane with the continuous microfibrils from the margo to the torus. Occasionally, the pit membrane of latewood tracheids in pine lacks the circular orientation of microfibrils on the torus surface. It was recently shown by Fujikawa and Ishida (1972) and was also observed in this study as shown in Fig. 38. It is supposed that the pit membrane of the relatively small size does not show the circular orientation of microfibrils on the torus, which is the characteristic of the bordered pit membranes of *Pinus* tracheids.

2.1.4. Formation of microfibrillar structure of the bordered pit membrane

Based on the work of a number of investigators, it is reasonable to assume that the bordered pit membrane of softwood tracheids originates from the primary wall (Frey-Wyssling et al. 1956; Fengel 1966; Bauch et al. 1969) and that the torus structure is not a drying artifact, as some workers has suggested. It is a real structure

produced during cell growth (Liese 1965; Tsoumis 1965; Thomas 1968; Dunning 1969b). The observations of the present study supply ample evidence for agreement with these points of view. But the process and mechanism of the formation of the microfibrillar structure of the torus and margo have not yet been fully explained.

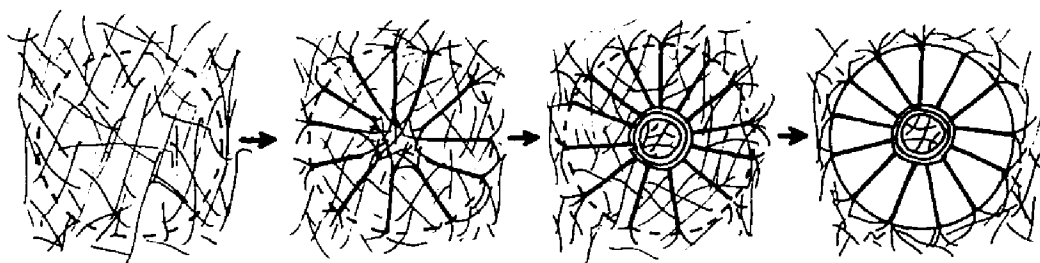
The bordered pit membrane consists of a perforated margo with variable density and an inperforated torus. The density variation of the margo appears to be controlled by the number of radially-oriented, large microfibrils and randomly-oriented, small microfibrils. In the bordered pit membrane, the radiating strand appears to consist of aggregation of a number of microfibrils. Thomas (1968) observed the small microfibrils aggregate into large strand in the periphery of the torus and in the annular region of the pit membrane.

Fig. 24 shows the inner surface of the tracheid in the very early stage of the development in which the cell dimension has reached its final size. The circular area is assumed to be the pit membrane developed from the primary pit field. The relatively small amount of microfibrils in the primary pit field, results in the small thickness of the pit membrane in this stage. This evidence appears to be in good agreement with the comment of Kerr and Bailey (1934) that during the elongation phase of cell growth, the primary wall is stretched and thus becomes thinner especially in the designated area of pit membrane development.

The pit membrane in relatively developed stage is shown in Fig. 25, in which the radiating microfibrils of the margo are deposited

on the primary wall microfibrils. The close examination of the figure makes it clear that the microfibrils underneath the radiating microfibrils take on the dense structure of the primary wall. The pit membrane in a little advanced stage takes on a wide separated network structure as shown in Fig. 13.

From the fact described above, the formation of the opening within the margo of a bordered pit membrane may be explained as follows; the radiating microfibrils are deposited in aggregation of the small microfibrils, and then existing microfibrils underneath the radiating strands are degraded by some enzymes such as cellulase. According to Thomas (1968), the degradation of the primary wall microfibrils, without removal of the radiating microfibrils, makes it necessary that they are structurally different. It is reasonable to assume that the degree of polymerization of the primary wall microfibrils increases as the formation stage proceeds (Marx-Figini, 1969). The formation of the microfibrillar structure of the bordered pit membrane is diagrammatically shown in Text-Fig. 4.



Text-Fig. 4 Diagrammatic representation of the development of the *Pinus* type bordered pit membrane.

2.2. Embedding substances of pit membrane and their degradation by enzymes

It was noted that the differentiating bordered pit membranes of coniferous tracheids were embedded in amorphous substances (Fengel 1966; Thomas 1968; Bauch et al. 1968). The chemical composition of the substances and the process of their removal in the last stage of development has never been fully understood. The first objective of this section is to examine their nature through chemical treatment and to observe degradation process of the amorphous substances in the margo.

It has also been noted that after the completion of the cell wall formation the embedding substances disappear and that the microfibrillar structure of the torus and the margo is exposed (Thomas 1968; Imamura, Harada 1973). The degradation of the embedding substances is assumed to be caused by the enzyme action by observations of the pit membranes during the perforation process (Thomas 1972; Imamura et al. 1974b). However, it is still remained to be solved whether the degradation is actually caused by enzyme action and why the margo region of the pit membrane is readily perforated leaving the torus region.

The question of the perforation of pit membranes may be solved by examination of differentiating pit membranes treated with enzymes hydrolyzing non-cellulosic polysaccharides. In this study, the bordered pit membranes treated with enzymes were compared with those developed under the natural condition in the living tree, and the perforation mechanism was discussed.

2.2.1. Chemical composition of the embedding substances in the margo and their removal in the living condition

It is obvious that the bordered pit membrane in conifer tracheids is embedded in amorphous substances throughout the differentiating zone. But their composition or chemical nature has been open to question, though it is fairly certain that they are non-cellulosic substances (Thomas 1968; Imamura et al. 1973). The appearance of the split surface extracted with 5% or 24% KOH is illustrated in Fig. 26 or Fig. 27, respectively. As the micrographs reveal, the outer surface of the pit border facing the pit chamber is not covered with the warty layer being visible through the interstice of the margo, and it is apparent that the bordered pit membrane has been in the differentiating zone. From the above fact and the observation that the warty layer is not removed by KOH as shown in Fig. 38, the pit membranes are supposed to have been fully embedded before the treatment. In Fig. 26 the embedding substances are partially dissolved, producing the small holes in the margo. In comparison with that, in Fig. 27 most of the amorphous substances are removed, leaving the skeleton of cellulose microfibrils of the margo. It is said that an extraction with 5% KOH removes the more soluble polysaccharides such as glucuronoxylans which are readily dissolved by dilute KOH solution (Browning 1967). It is difficult to determine the chemical nature of the substances precisely, but it is plausible that they are composed of some kind of non-cellulosic polysaccharides having varying solubilities in KOH solution. This evidence is supported by the

staining reaction using safranin-astrablue (Bauch et al. 1968). The pit membrane in the boiled sample (Fig. 28) shows the partial removal of the embedding substances. The pit membrane found in Fig. 28 was derived from an early stage of the differentiating tracheids, but the one in a more advanced stage showed no detectable change in the margo region. This observation coincides with the fact that the substances in the very early stage are fully removed by 5% KOH solution, and that in an advanced stage 24% KOH solution is necessary for removal of the substances. It is assumed from these results that hemicelluloses soluble in hot water are deposited in the margo in the stage of the primary wall formation and that other kinds of hemicelluloses which are hardly soluble in the dilute KOH or hot water are added with continued development.

The perforation process of the bordered pit membrane which occurs in the last stage of its development is not fully understood. Thomas (1968) showed that perforated pores are first visible in the vicinity of the torus. Imamura and Harada (1973) noted that the perforation did not begin until the tracheid wall was completely formed. It was noted by Thomas (1968) that the observation of the pit membrane in the degradation process of the margo amorphous substances was difficult. In this study the difficulty was overcome by using the sample which had been frozen and fixed immediately after cutting from the living tree. It is observed that degradation of the amorphous substances initiates at the surface (as shown by arrows), and then proceeds into the inner part of the depth of the margo (Fig. 29). From the

appearance of the small opening near the torus, it is supposed that they have been formed not mechanically but by enzymatic action. In the bordered pit membrane which is in the more advanced stage of differentiation, the amorphous substances appear granular and are attached to the margo microfibril (Fig. 30). In an almost perforated pit membrane, the amorphous substances are only recognized in the margin of it (Fig. 31).

2.2.2. Enzymatic degradation of the embedding substances of the margo

It has been noted that the degradation of the amorphous substances, which embed the margo of the bordered pit membrane throughout the differentiating xylem zone, begins in the vicinity of the torus after the completion of the cell wall development (Thomas 1968; Imamura, Harada 1973). It has also been observed that the degradation begins at the surface of the pit membrane and then proceeds to the inner part. In a more advanced stage of degradation, the amorphous substances appear granular, and are attached to the margo microfibrils.

In this study, samples containing pit membranes, which were fully embedded in amorphous substances (Figs. 14, 19), showed distinct morphological changes in the margo region after the hemicellulase treatment (Figs. 32-34). The control sample treated in the enzyme free solution resulted in the intact pit membrane and did not show any notable changes when compared with untreated samples.

A slight degradation of the bordered pit membrane by the action of hemicellulase is shown in Fig. 32. The erosion of the embedding

substances is shown to begin at the surface of the pit membrane, but no openings are visible. In a more advanced stage of degradation, the interstices are seen between the radiating microfibrils in the margo (Fig. 33). These micrographs indicate that the digestion with hemicellulase begins at the surface and proceeds to the inner part of the margo. Fig. 34 shows an almost completely perforated pit membrane with a small amount of the embedding substances still attached to the margo microfibrils, disclosing the same microfibrillar structure as a mature pit membrane. This micrograph shows the pit membrane structure to be identical to that treated with Jeffrey's solution or 24% KOH solution, as reported in the foregoing sections.

From the observations shown in Figs. 32-34, the breakdown of the embedding substances by enzyme treatment appears to be performed in essentially the same manner as natural degradation in the living tree which is presented in the section 2.2.1. Thus, it is reasonable to consider that the margo is perforated by the action of enzymes, such as "hemicellulase", after the cell wall formation has completed.

The influence of wood-destroying fungi and microbial enzyme on the structure of wood has been reviewed by Cowling (1965). A major advantage of using an enzyme system is the very mild condition under which its activity proceeds. Wood et al. (1952) used pectinase to make the primary wall microfibrils visible, without employing rigorous extraction treatment. It was recognized in the present study that the degradation initiates at the surface and proceeds gradually into the inner part of the membrane. Due to the mildness of the

digestion reaction, pit membranes in various stages of degradation and those without any severe damage to the cellulose microfibrils could be observed. The mild process of the enzyme action may be assumed to result from the fact that enzymes are large protein molecules and, as such do not diffuse readily into the pit membrane but spread slowly from the surface to the inside, gradually digesting the materials.

Another characteristic of enzyme reaction is of high specificity to the substrate. It has been noted that the treatment of wood with several enzymes is one method of obtaining information on its chemical nature (Suolahti, Wallén 1958; Cowling 1965; Nicholas, Thomas 1968). This method of enzyme treatment indicates that the embedding substances of the margo in the differentiating pit membranes are composed of hemicelluloses. Pure enzymes which are difficult to obtain, have not been used, and then it is not easy to determine precisely the chemical properties of sample by enzyme treatment alone (Reese, Mandels 1959). The concept that the embedding substances are like hemicelluloses in composition is, however, more in agreement with the histochemical results.

It was observed that the degradation of the embedding substances of the margo in a series of the differentiating tracheids did not proceed in the same manner during the enzyme treatment of pit membranes. The result is well agreement with the proposal that the chemical composition of the substances changes as the developmental stage proceeds.

2.2.3. Resistance of the torus to the perforation

The pit membrane in the differentiating zone of a sample which has been treated with pectinase is shown in Fig. 35. Although no notable change can be detected in the margo, the future torus region is degraded by the enzyme action. Nicholas and Thomas (1968) observed that pectinase caused a considerable degradation of the torus in mature sapwood, indicating a high pectin content in the torus. From an observation of Fig. 35 it is evident that, even in the differentiating pit membrane, the pectin content in the central part, the future torus region, is higher than in the periphery area of the membrane, the margo. Erosion in the torus region by the pectinase treatment was commonly observed in the species studied here.

In this micrograph (Fig. 35), the center of the pit membrane is completely eroded, and the microfibrils which should remain in this area if pectinase degrades only pectin substances, are not found. It is supposed that this area is eroded so severely that the surviving microfibrils are distorted in the drying or replication process.

Thomas (1972) proposed that some mechanism must be operative to prevent enzymatic action in the future torus region. He also noted that the concentrically deposited microfibrils in the torus block its action in pine, and that abundant amorphous materials on the torus has a similar effect in cypress (*Taxodium distichum*). He thought that in cypress the substances constituting the thickened torus were the same as the embedding substances in the margo, and that both substances

were simultaneously removed by the same enzyme action. Fengel (1972) also supposed that the enzyme acted upon the whole membrane and that the torus only remained, because this part of the membrane was thicker than the margo. O'Brien (1970), on the other hand, suggested that the polyuronide-rich materials added to the pit membrane during formation of the torus may produce a membrane with a varying degree of resistance to the hydrolysis.

It was observed that the differentiating pit membrane of *Cryptomeria japonica* (Fig. 36) or *Chamaecyparis obtusa*, which does not have circularly oriented microfibrils or additional amorphous substances, as are present in the *Pinus* or *Taxodium* species, was degraded only in the margo region by the hemicellulase treatment. This observation makes it difficult to assume that only the additional microfibrils or amorphous materials protect the torus from the enzyme degradation, as noted by Thomas (1972) and Fengel (1972). Incidentally, pectinase degraded the torus region in the differentiating pit membrane from these species as well as in that from *Pinus* tracheids. From these results, O'Brien's suggestion that the abundant pectin substances in the torus resist the enzyme action which degrades the embedding substances in the margo, is fairly reasonable.

2.2.4. Incrustation of the margo in the mature condition

The observations of the pit membrane development presented above were done using earlywood tracheids. The development of the latewood bordered pit membrane was also examined. The bordered pit membrane of

the latewood tracheid is also embedded in the amorphous substances showing the same appearance as that of earlywood tracheids throughout the differentiating zone. However, even in the mature condition the bordered pit membrane of the latewood is incrustated with amorphous substances (Fig. 37).

The nature of the incrusting substances of the latewood bordered pit membrane has been unknown. In this study they were removed by 24% KOH extraction (Fig. 38), and also by hemicellulase treatment (Fig. 39), leaving skeletons of cellulose microfibrils. From these results, it is clear that the chemical nature of the incrusting substances of the mature latewood pit membrane is similar to that of the embedding substances (hemicelluloses) of the differentiating pit membrane. It was supposed that the amorphous substances which embedded the differentiating pit membrane were not completely removed in the last stage of the formation.

It is interesting that the incrustated pit membrane of mature latewood (Fig. 37) shows the very similar appearance with the differentiating earlywood pit membrane in the degradation process (Fig. 29). A hypothesis is proposed that the activity of the enzyme decreases in the stage of latewood formation, and that, as a result some embedding substances of the differentiating pit membrane survive after the enzyme action has ceased. From Petty and Puritch (1970) the degree of incrustation of the mature latewood pit membrane in any annual ring increased from the first-formed tracheid to the last-formed one, suggesting the decrease of enzyme activity in the last

formed region.

The pit membranes of *Abies* species possess so-called "torus extensions" (Krahmer, Côté 1963; Bauch et al. 1972, Fujikawa, Ishida 1972) which include amorphous substances (Fig. 40). The differentiating pit membranes from this species were also thoroughly embedded in amorphous substances, showing a similar appearance to those found in other species. The hemicellulase treatment of this species removed embedding substances, leaving no torus extensions but exposing the microfibrils of the margo (Fig. 41). This fact suggests that there is little difference in the chemical nature of the embedding substances among these species, and the embedding substances survive in this species after maturation because of the inhomogeneity of the enzyme action.

2.2.5. Development of the perforation plate in hardwood vessels

The development of the perforation plates in vessels of hardwood has also been studied by several workers (Esau, Hewitt 1940; Yata et al. 1970; Meylan, Butterfield 1972). The primary walls and middle lamella in the region of the perforation remain intact late in the differentiation, being embedded in the amorphous substances. After the secondary wall has been laid down on the other areas of the vessel walls, the perforation partitions are broken down. Butterfield and Meylan (1972) showed the scanning electron micrographs of them in the perforation process and supposed that the breakdown occurred by the enzyme action. The development of the perforation plate presented by

them is very similar to that of the bordered pit membrane of coniferous tracheids.

These perforation plates are, however, relatively simple openings in the cell wall compared with the more complex structure of pits where a separating "membrane" may still be present. It has been yet uncertain whether microfibrils are removed enzymatically or passively from the perforations. O'Brien (1970) noted that if the loss of cellulose is achieved passively (e. g. by the transpiration stream), the difference of the perforation mechanism between tracheids and vessels would readily be determined by the same pattern.

Fig. 42 shows the scalariform perforation partition in the differentiation condition from *Cercidiphyllum japonicum*. The perforation plate is fully embedded in the amorphous substances. The treatment with hemicellulase removed only the amorphous substances, revealing the dense structure of the primary wall microfibrils (Fig. 43). Incidentally, the hemicellulase treatment of the differentiating bordered pit membrane of conifer tracheids showed the perforated structure of microfibrils as the mature one. Considering the difference between the bordered pit membrane and perforation plate, it is assumed that the perforation mechanism in the living tree is not the same between the two.

Observations of the perforation plate in the digestion process established that the amorphous substances and cellulose microfibrils are simultaneously degraded (Figs. 44, 45). It is supposed that two kinds of enzymes are simultaneously operative in the degradation

process of the perforation plate, digesting the non-cellulosic polysaccharides and cellulose microfibrils.

2.2.6. Experimental

(1) KOH extraction Split radial sections containing the differentiating xylem were immersed in the solution of 5% or 24% potassium hydroxide (KOH) for overnight at room temperature.

(2) Hot water extraction Sections were boiled for 3 hours in a bath.

(3) Enzyme treatment Enzyme solutions were prepared by dissolving 0.4 gr. of hemicellulase (Sigma, *Rhizopus mold*) in 20ml of veronal citrate buffer (pH: 5.5), and 0.1 gr. of pectinase (Sigma, *Aspergillus niger*) in 20ml of citrate buffer (pH: 4.6), respectively. Three specimens were placed in 20ml of each of the enzyme solutions containing 0.1 gr. of dehydroacetate to prevent growth of microorganisms, and incubated at 45°C (hemicellulase) and at 35°C (pectinase) for 3 or 5 days.

As control, samples were also treated in enzyme-free buffers.

After each treatment, the samples were washed with distilled water and then freeze-dried.

2.3. Development of the pit border

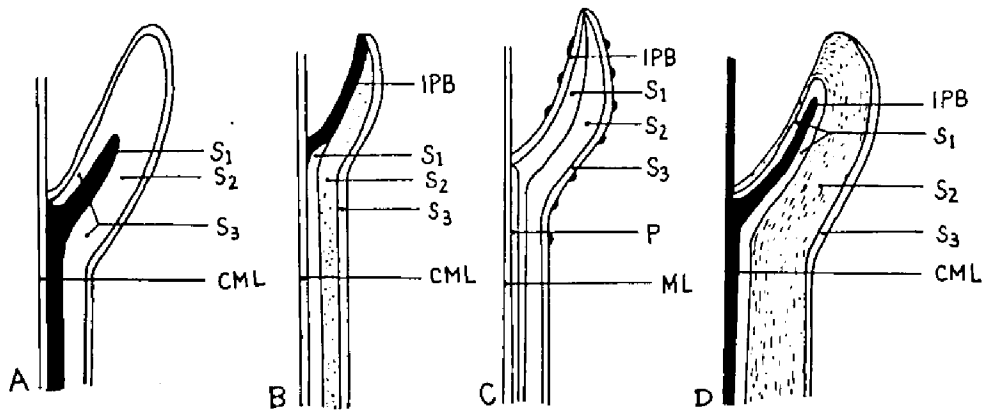
The cell wall organization in the pit border region has been studied from the view point of its structural variation.

Since the investigation of the organization of the cell wall in

the region of the pit border of coniferous tracheids by optical microscopy of Bailey and Vestal (1937), many publications in this field appeared with introduction of electron microscopy. The point of argument on the cell wall organization in the pit border region is whether the layer having concentric microfibrillar orientation should be distinguished from the S_1 -layer or whether the cell wall organization of the pit border region is different from that of the unpitted region.

According to Wardrop and Dadswell (1957), Fengel (1966), and Murmanis and Sachs (1969), the deposition of the cell wall layer in this region begins by the deposition of concentrically oriented microfibrils, the so-called B.T. or initial border thickening, followed by the S_1 , S_2 , and S_3 -layer deposition. However, it seems unreasonable to consider that the B.T., which has different microfibril orientation from that of the primary wall, is formed only in this region before the deposition of the secondary wall. Although many publications on the structure of the pit border region have appeared (Wardrop, Davies 1961; Jutte, Spit 1963; Fengel 1966; Harada, Côté 1967; Murmanis, Sachs 1969), hardly two descriptions on the structure of the border of coniferous tracheids are completely identical (Text-Fig. 5). For example Jutte and Spit (1963) interpreted a contrasting zone in the pit border to be the S_1 -layer, but Murmanis and Sachs (1969) thought this to be the primary wall. On the other hand, Harada and Côté (1967) showed the micrograph of a tangential section of the pit border with scale-like appearance in the S_1 -layer, but they did not propose

any explanation regarding this pattern.



Text-Fig. 5 Comparison between diagrams representing the structure of the pit border. A, Wardrop and Davis (1961); B, Jutte and Spit (1963); C, Harada and Côté (1967); D, Murmanis and Sachs (1969). A, B, C for earlywood, D for latewood.

The purpose of the present work is to obtain information on the process of cell wall development in the bordered pit region through observation of the inner surfaces of the cell wall in differentiating tracheids.

2.3.1. Earlywood bordered pit

S₁-layer

Before the inner surface of the primary wall is formed, the circularly

oriented microfibrils have been laid down making the periphery of the pit area. Then the development of the pit border is started by the formation of the inner surface of the primary wall (Fig. 13). The transversely and wave-like oriented microfibrils of this layer in an unpitted area show their circular orientation around the developing pit aperture. Subsequent to the development of the pit border with the growth of the inner surface of the primary wall, the deposition of the outer layer of the secondary wall occurs at the pit border.

Fig. 46 illustrates developing pit border of the tracheid on which the S_1 -layer is depositing. In this micrograph a pit membrane can be observed through a large pit aperture, and also the outer surface of the pit border of the adjacent tracheid can be seen through the interstice of the pit membrane torn off. The visualization of the pit border of the adjacent tracheid can be attained by the fact that the two neighbouring tracheids are not equivalent to each other in the stage of development. This micrograph also demonstrates both the microfibrillar orientation of the S_1 -layer on the inner surface of the pit border in the tracheid of the one side, and the concentric pattern of microfibrils on the outer surface of the pit border in the adjacent tracheid. Therefore the concept that the formation of initial border thickening having the concentrically oriented microfibrils begins before the deposition of the S_1 -layer cannot be accepted from this observation. In other words, the S_1 -layer contributes to the pit border development from the tracheid lumen side corresponding to the formation of concentrically oriented microfibrils on the outer

surface of the pit border.

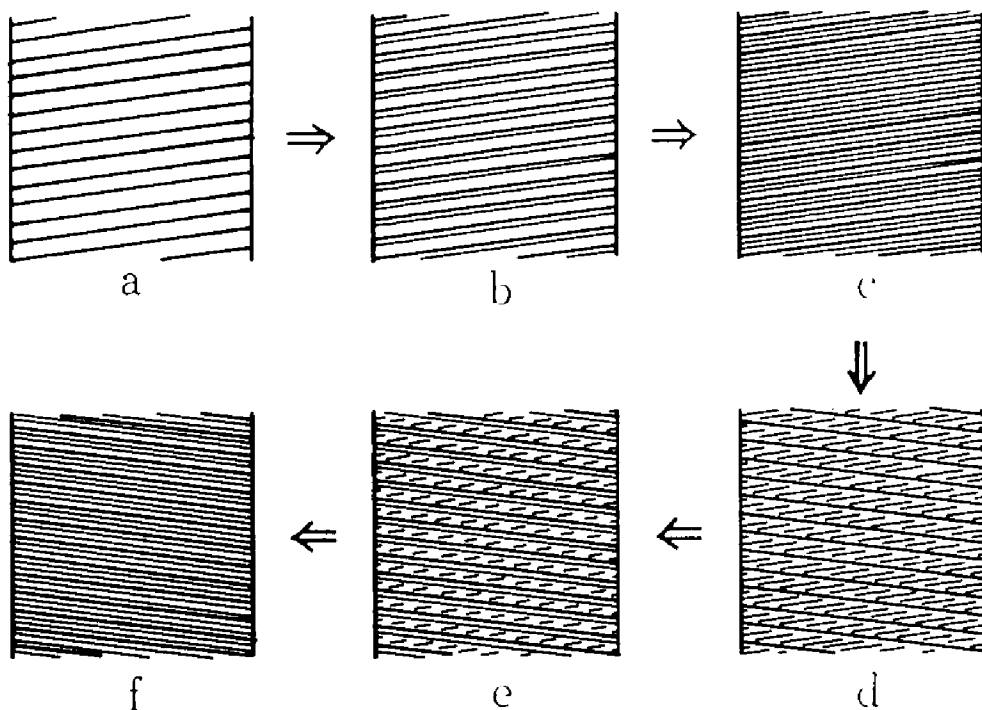
The S_1 -layer in the pit border region can be distinguished from the initial pit border thickening by both their microfibrillar orientation and their appearance revealed in the cross-sectional view, and both the S_1 -layer and initial border thickening are assumed to be formed supplementally each other. So the concept, suggested by Wardrop and Dadswell (1957), that the initial border thickening having concentrically oriented microfibrils concludes its development prior to the secondary wall deposition, is denied. Therefore it does not seem proper that the layer having concentrically oriented microfibrils is termed "initial border thickening". However this term is conventionally used in this paper.

In the pit border of the developing phase of the S_1 -layer, the newly deposited lamella can be detected to be crisscrossed with the underlying lamella having the almost transversely oriented microfibrils (Fig. 47).

Fig. 48 also shows the developing pit border in the stage of S_1 -layer formation. The transversely deposited microfibrils in the pit border region generally curve around the pit aperture and some of them make a detour to the outer surface covering the tip of the border.

The formation of the S_1 -layer is supposed as follows; in the inner surface of the developing wall, microfibrils are found to be deposited to form the "microlamella" in which the microfibrils lie parallel to each other (Imamura et al. 1972b). Each microfibril

keeps a constant distance of about 700-800Å (Fig. 49). The progressive development of successive microlamellae was observed in the surface of one tracheid forming the S_1 -layer (Fig. 50). This observation confirms the hypothesis of Wardrop and Harada that the secondary wall formation begins near the middle of the fiber and proceeds towards the ends. In the S_1 -layer if some microlamellae of the same microfibril orientation are deposited, a simple helical orientation will be



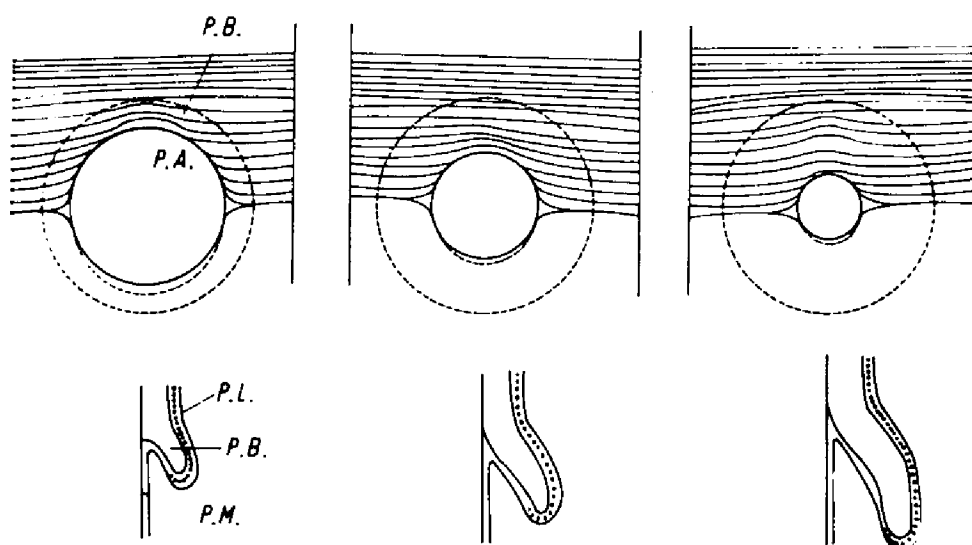
Text-Fig. 6 Diagrammatic representation of the deposition of microlamellae in the S_1 -layer from the surface view.

detected (Text-Fig. 6c). As the microfibrils of a microlamella keep a wide distance to each other, the newly formed one of the same orientation could be deposited between the former ones (Text-Fig. 6a-b-c). But if newly deposited microlamellae go through a small change in orientation, the crisscrossed structure will be observed (Text-Fig. 6d). Though several microlamellae are deposited in the same direction as the new one, a simple helical orientation will appear again (Text-Fig. 6f). So the crisscrossed appearance is observed, not only because successive lamellae have alternating right and left-hand spirals, but also because the newly deposited microlamellae have the small changes in fibril orientation from the underlying lamellae.

A lamella formed by successive deposition of some microlamellae of the same microfibril orientation is supposed to be equivalent to "an elementary lamella" noted by Heyn (1969). He defined an "elementary lamella" as the thinnest cellulose lamella consisting of one single layer of elementary fibrils.

These observations present an assumption regarding the process of pit border development. A model based on the above assumption showing both the orientation of microfibrils and the process of pit border development in the stage of the S_1 -layer deposition is shown in Text-Fig. 7. The model explains that the diameter of the pit aperture is large in the early stage of development and that it decreases gradually with the deposition of the S_1 -layer. It has been also shown from Text-Fig. 7 that the development of the pit border is made when deposition of the new microlamella of the S_1 -layer proceeds,

and that in this case, however, the microfibrils which are producing the microlamella in the S_1 -layer curve through the inner edge of the pit aperture onto the outer surface of the developing pit border. Consequently the amount of curved microfibrils decreases with the growth of the pit border.



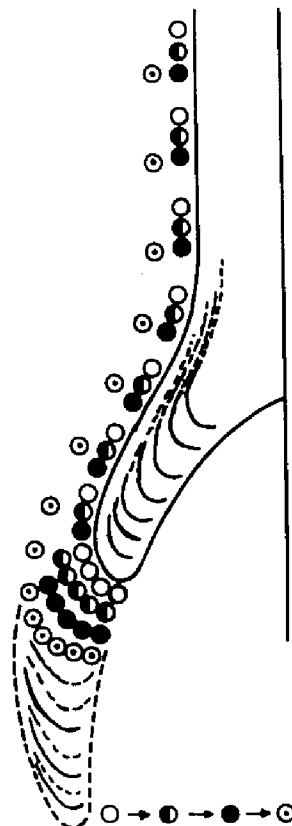
Text-Fig. 7 Diagrammatic representation of the pit border development in the stage of the S_1 -layer formation. Upper and lower show the surface and sectional view respectively. PB: pit border, PA: pit aperture, PL: plasma membrane, PM: pit membrane.

The assumption of the pit border development is supported by the observation of the replica shown in Fig. 50. In this pit membrane the microfibrils which should extend over the top and outer surface of the pit border were pulled out to the tracheid lumen, covering the open of the pit aperture. The movement of microfibrils is assumed to

be brought out by migration of the cell component by plasmolysis.

The depositing microfibrils of a microlamella which lie parallel to each other in the unpitted region, aggregate and form a scale in sectional view of the pit border. In Text-Fig. 8, the microlamella composed of the microfibrils shown by (○) are deposited at first, then the second microlamella dotted by (◐) is deposited. Though the

Text-Fig. 8 Diagrammatic representation of the deposition of microlamellae in the S_1 -layer from the sectional view. Microlamellae contribute to the scale-like texture in the pit border. Each dot shows a microfibril.



microfibrils of the later microlamella lie between those of the former one in the unpitted region, two scales are formed in the pit border (Text-Fig. 8). The number of scales of the pit border is, so that, assumed to correspond to that of the microlamellae in the S_1 -

layer.

Fig. 51 is a tangential section of differentiating tracheids stained with KMnO_4 . In this micrograph the scale-like texture can be observed in the pit border region. A micrograph similar to that shown in Fig. 51 was presented by Harada and Côté (1967) using the shadowed section of the mature tracheid of *Pinus taeda*. Such a pattern in the pit border can be explained from the process of S_1 -layer formation as shown in Text-Fig. 7. The number of scales which are observed in the cross sections is ca. 100, and this number is assumed to be corresponding to that of the microlamellae of the S_1 -layer.

In order to ascertain the process of microfibrillar deposition in the S_1 -layer, the model of microfibrillar orientation in the pit border region is made using plastic tubes to represent microfibrils following the concept described above (Fig. 52). In this model the microfibrils, which are deposited almost transversely in the unpitted area, sweep around the pit aperture and take a detour over the tip of the border making the pit border develop. Then a number of microfibrils which should occupy the area of the developing pit aperture is supposed to extend over the outer surface of the pit border.

S_2 -layer

Following the S_1 -layer, the S_2 and S_3 layers are deposited in the tracheid wall. The orientation of microfibrils of the S_2 -layer around the pit border region was reported by Harada et al. (1958). The microfibrils of the S_2 , which were arranged in a more or less parallel orientation in the unpitted area, sweep around the pit

aperture in a stream line pattern (Fig. 53).

Incidentally, the detailed examination of Fig. 54 makes it clear that microfibrils are deposited through "microlamella" even in the S_2 -layer. As the change of angle between one microlamella and next one is relatively small, it is difficult to detect the lamellar structure in the unpitted wall.

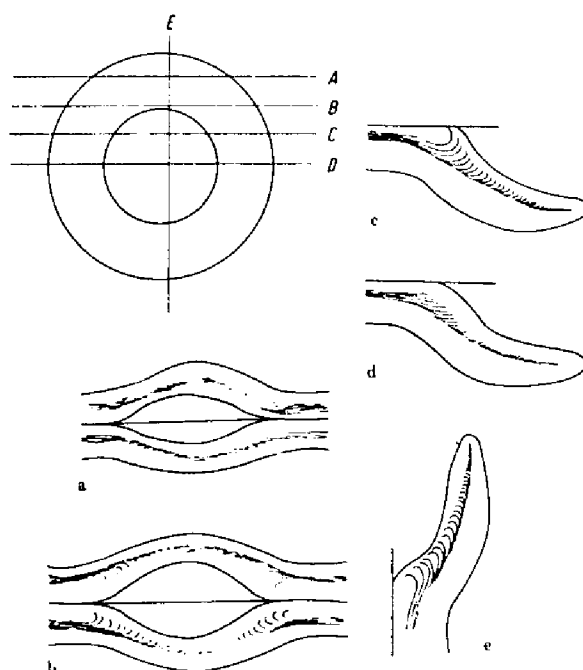
As the pit border in the stage of the S_2 -layer formation is well developed and protrudes of great degree into the tracheid lumen, it appears unnecessary for microfibrils of a microlamella which should cover the pit aperture, to extend to the outer surface of the pit border.

Organization of the pit border

Studying the developmental sequence of the pit border, the pit border model proposed by Harada and Côté (1967) is more plausible. According to them, the secondary wall deposition stops at the tip of the border, and the outer surface of the pit border is covered by the initial border thickening.

If the pit border is formed through the process mentioned above, the appearance of the pit border in cross section would change depending on the plane of sectioning (Text-Fig. 9). In this figure, if the pit border is cut through the line (A), (B), (C), (D) and (E), it would be shown as in (a), (b), (c), (d) and (e) respectively. The micrograph showing a scale-like appearance would be observed, if the section is obtained within the range of the line (B) to (D) and

near the line (E).



Text-Fig. 9 Proposed diagram concerning the cross sectional view of the pit border of the earlywood tracheid in various cutting planes.

Observations of the ultra-thin sections cut at several levels show the appearances in agreement with the above assumption. Fig. 51 is assumed to correspond to (e) in Text-Fig. 9, and Fig. 55 to (a), respectively.

Murmanis and Sachs (1969) have examined the radial sections of the tracheid wall in the pit border region, and have noted that they do not reveal much of the different wall layer organization and are considerably more difficult to interpret. The radial section was prepared in this study and was allowed to be explained following the

above description on the pit border development (Fig. 56).

In the ultra-thin section stained with KMnO_4 (Fig. 51), the part of a scale-like appearance exhibits electron density and is assumed to be rich in lignin content. Jutte and Spit (1967) also observed a contrasting zone in the pit border and interpreted the dark zone to correspond to the S_1 -layer. Murmanis and Sachs (1969) thought this zone to be the initial pit border of the primary wall origin. UV microscopy has also been instrumental in showing a similar region in black spruce (Fergus et al. 1969). The lignin-rich zone in the pit border was also shown by residual lignin skeletons prepared by acid hydrolysis of the wood carbohydrates (Sachs et al. 1963; Parham, Côté 1971). It is assumed that this region is developed through a successive deposition of microlamellae of the S_1 -layer and is porous in the formation stage resulting in the lignin-rich condition.

2.3.2. Latewood bordered pit

The shape of pits shows a gradual change from earlywood to latewood, viz. a circular to a lenticular aperture. The typical latewood pit shows a so-called extended pit aperture (Fig. 57), in which the inner aperture is larger than the outer aperture. It is supposed that the developmental sequence of the latewood pit border is not equal to that of the earlywood one.

Pit border development of the latewood was detected to be initiated by the deposition of circularly oriented microfibril around the pit annulus in the formation stage of the primary wall. More development

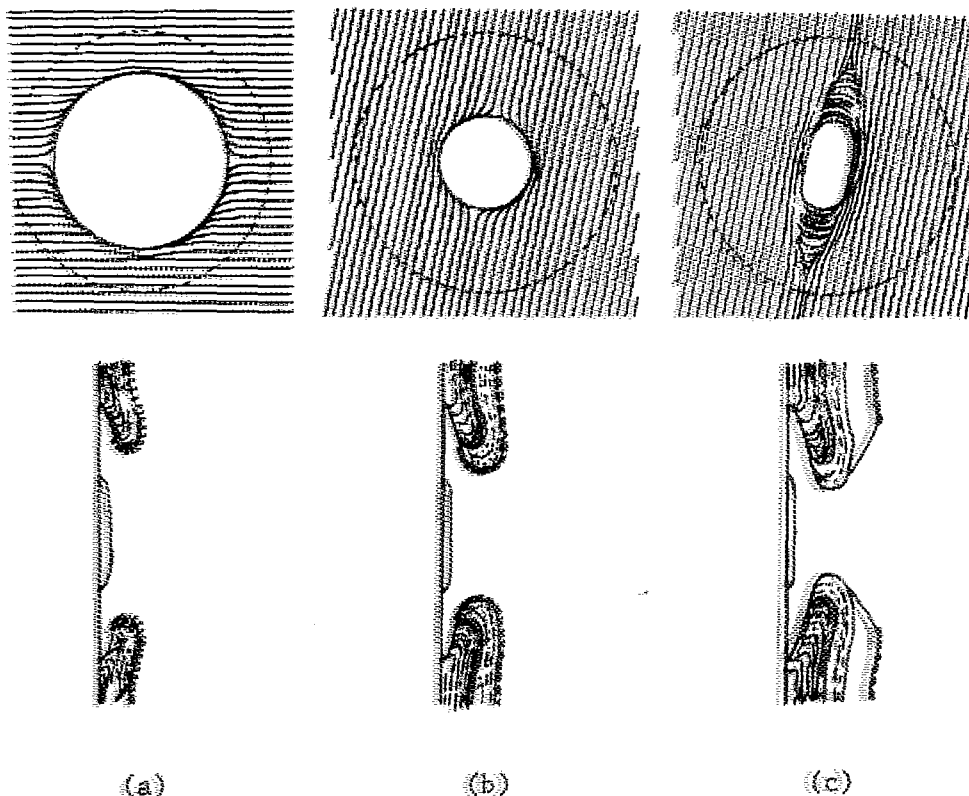
of the pit border is progressed by the deposition of microlamellae of the S_1 -layer in the tracheid lumen side and concentrically oriented microfibrils in the pit chamber side. The formation manner until this stage is very similar to that of the earlywood pit border.

While in the earlywood tracheid the S_2 did not concern with pit border elongation, in the latewood tracheid the microfibrils of the S_2 -layer were observed to curve around the pit aperture and some of them to extend to the top of the developing pit border (Fig. 58). This micrograph shows a similar pattern of microfibril deposition in the S_2 -layer to that in the S_1 -layer described previously. In this micrograph, the microfibrils curve around the pit aperture and some of them extend to the outer surface of the pit border beyond the top of it.

If the pit border of latewood is formed in such manner as was observed in the developing pit border of earlywood, it is expected that the scale-like pattern should appear in the S_2 as well as in the S_1 in sectional view of the latewood pit border. But the observation of the transverse or tangential section of the latewood pit border prepared by $KMnO_4$ staining or metal shadowing established that the pattern appears only in the outer part of the border (Murmanis, Sachs 1969; Siau 1971). So it is suggested that the pit border with extended pit aperture is formed by the identical manner described above in the early stage of the S_2 -layer formation, but not in the late stage of its formation. As a matter of fact, in the later stage of the layer formation, the microfibrils do not extend to the

developing pit border, but sweep only around the pit aperture in a stream like pattern (Fig. 59). Simultaneously concentrically oriented microfibrils are deposited around the outer aperture in arranging its shape.

A schematic representation of pit border development of the latewood tracheid is shown in Text-Fig. 10. In this diagram (a) indicates the microfibril orientation of the S_1 , showing the same texture as that in the earlywood tracheid. The contribution of the S_1 -layer to pit border development ends halfway before the outer pit aperture reaches its final size. Following the S_1 -layer deposition,



Text-fig. 10 Diagrammatic representation of pit border development of the latewood tracheid, upper and lower show the surface and sectional view, respectively.

the S_2 -layer contributes to the pit border formation. (b) shows the microfibril orientation in the early stage of the S_2 -layer formation, which makes the outer aperture into the final shape. A representation of the microfibril orientation in the late stage of the S_2 -layer formation is shown in (c), while the inner aperture is formed into the final shape. The corresponding explanation of cross sectional views are shown in the lower diagrams respectively. Although the electron micrographs of ultrathin sections of latewood pit border have been presented, the cell wall organization has neither been described correctly, nor has the appearance been explained. The surface observations in this study give a good explanation of the organization.

2.4. Summary

The observation of the inner surface of the coniferous tracheid walls in various stages of the differentiation allowed the study of the development of the bordered pit.

The process of the formation of the bordered pit membrane was examined in detail using mainly *Pinus densiflora*, *Cryptomeria japonica* and *Chamaecyparis obtusa*. The microfibrillar structure of the margo and torus was detected in the later stage of the primary wall formation. The bordered pit membrane was shown to have the same microfibrillar structure as that of the matured one even at this stage of differentiation, although it was embedded in matrix substances. It was observed that the matrix substances were removed

immediately after the warty layer had been formed in the tracheid wall. The evidences presented above were recognized to be applicable to the bordered pit membrane of various coniferous species and to that between ray-longitudinal tracheids.

The embedding substances of the differentiating pit membrane are assumed to be some hemicelluloses, which are removed by enzyme action. In order to ascertain their perforation mechanism, the pit membranes of the differentiating tracheids were treated with hemicellulase and pectinase, and the morphological changes caused by the enzymes were examined. Hemicellulase degraded only the embedding substances of the margo, whereas pectinase caused digestion in the future torus region. These results make it realistic that the embedding substances are degraded by the enzyme such as hemicellulase and that the torus is resistant to the enzyme action by abundance of pectin materials in the development.

The method for observing the inner surface of differentiating tracheids was developed in order to study the formation and organization of the cell wall in the pit border region. The initiation of pit border development was observed as the deposition of circularly oriented microfibrils around the vicinity of the pit annulus in the later stage of the primary wall formation. In earlywood tracheids, the deposition of the S_1 -layer was indicated to contribute to the pit border formation until the diameter of the pit aperture reached its final size, simultaneously with the apposition of the layer of so-called B. T. or initial border thickening. So both the S_1 -layer and

initial border thickening were assumed to be formed supplementally each other. In the period of the S_1 -layer formation, the transversely deposited microfibrils in the tracheid inner surface were detected to generally curve around the pit aperture and some of them extend to the outer surface of the developing pit border beyond the pit aperture. In the pit border of latewood tracheids, the S_2 -layer as well as S_1 was recognized to contribute to the pit border development. From these observations of replicas of the inner surface of the differentiating tracheid, an explanation concerning pit border formation is proposed.

3. Development and ultrastructure of the pits of parenchyma cells

3.1. Development of the pits of thin-walled ray parenchyma cells

Pits of ray parenchyma cell is not less important than those of tracheids, when considered as the passage of liquids between tracheid and ray cells or as the channel in radial direction. They are of considerable interest as modified regions of the cell wall organization. Although many works have appeared regarding the bordered pit membrane structure interconnecting the longitudinal tracheids, only a few studies have been done on the pit structure in ray parenchyma cells of softwoods.

Several workers (Harada 1953, 1964; Frey-Wyssling et al. 1956; Krahmer, Côté 1964; Thomas, Nicholas 1968; Fengel 1970) have focussed attentions on the structure of the cross-field pitting that interconnects the longitudinal tracheid and the ray parenchyma cell. As a result, the pit membrane was found to consist of the primary wall of the tracheid and the complete wall of the ray parenchyma cell. It was also indicated that both sides of pit membrane reveal the randomly oriented microfibrils embedded in amorphous substances, showing no detectable pores. Côté (1958), however, in the tracheid lumen surface of a cross-field pit membrane from *Pinus strobus* detected a particular microfibrillar arrangement at the margin of it, which was different from that of the ordinary primary wall. In addition to the inconsistency of the pit membrane structure on the tracheid side, the wall organization of the pit membrane on

the ray parenchyma side is still unknown.

Pits in both the transverse and end wall of a ray parenchyma cell, when present, are simple. As viewed in a radial section, the pits of some species exhibit conspicuous nodules while others do not (Panshin, de Zeeuw 1964). It has been said that *Pinus*, *Cryptomeria* and *Chamaecyparis* possess thin-walled ray parenchyma cells. Whereas *Abies*, *Pseudotsuga*, *Tsuga* and *Picea* have thick-walled ray parenchyma cells. The samples used in this section are confined to the species which have thin and smooth walls. Harada (1965) observed the transverse section of ray parenchyma cells of *Cryptomeria japonica* using electron microscopy and noted the gap or recess in the transverse wall which is assumed to be a simple pit. Thomas and Nicholas (1968), on the other hand, reported that in four species of yellow pine studied the end walls of the ray parenchyma cells were devoid of simple pits, presumably because of the absence of the secondary wall thickening. The structure of the simple pit, when found, is still not fully explained.

In this study, wood blocks containing earlywood differentiating zones were obtained from *Pinus densiflora*, *Cryptomeria japonica* and *Chamaecyparis obtusa*. Replicas were prepared from split radial sections in order to observe the inner surface of the ray parenchyma cells. For the examination of the cross-field pit membrane from the tracheid lumen side, microtomed radial sections are also used.

3.1.1. Pit membrane of cross-field pitting on the tracheid side

The shape, size and arrangement of pits in the cross-fields vary in different softwoods and therefore are of considerable importance in their identification (Panshin, de Zeeuw 1964). The samples used in this study revealed window-like, taxodioid and cupressoid pit types respectively.

It is apparent that the microfibril organization of the pit membrane of the tracheid side is inconsistent with the earlier concept of the random orientation of microfibrils. In *Cryptomeria japonica* and *Chamaecyparis obtusa*, the membrane exhibits radiating microfibrils in its periphery, showing an appearance similar to that generally found in the bordered pit membrane interconnecting the longitudinal tracheids (Fig. 60). The window-like pit membrane of *Pinus densiflora* shows the radial strands of microfibrils only in the margin of the membrane as shown by Côté (1958). Therefor the radiating structure of microfibrils is assumed to be a characteristic found generally in the tracheid side of the pit membrane between the tracheid and the ray parenchyma cell. It is of interest that even the cross-field pit membrane, which is supposedly not subject to aspiration, reveals radiating microfibrillar structure.

Development of the microfibrillar structure of the pit membrane was examined through the observation of the inner surface of the tracheid. Fig. 61 shows the tracheid in a late stage of primary wall formation which is evident from the wavy orientation of microfibrils in the unpitted area. The radiating structure of microfibrils is already evident in the area surrounded by circularly oriented

microfibrils (shown by arrows), which is assumed to be the initial stage of pit border development. Thus, the microfibrillar structure of the cross-field pit membrane is fully developed in the later stage of primary wall formation, following the same sequence as that found in the development of the bordered pit membrane interconnecting longitudinal tracheids (Imamura, Harada 1973; Imamura et al. 1974a).

3.1.2. Pit membrane of cross-field pitting on the ray parenchyma cell side

The differentiating tracheids constitute a radial file, which shows a sequential developmental stage of the primary wall, S_1 , S_2 and S_3 -layer, from cambium to maturity. It is difficult, however, to observe the inner surface of ray parenchyma cells in various developmental stages as found in the tracheids, since the number of the ray parenchyma cells in a series in the differentiating zone is less than that of tracheids. This difficulty was overcome by observing ray parenchyma cells of many specimens in the developmental stage.

The evidence obtained in *Chamaecyparis obtusa* is assumed to be applicable to the other two specimens. Fig. 62 shows the inner surface of a ray parenchyma cell in a very early stage of cell wall formation. Microfibrils run along the longitudinal direction of the cell, but are relatively interwoven. The microfibrils are more dispersed in the area where abundant pores originated from plasmodesmata can be seen. This area is assumed to develop into cross-

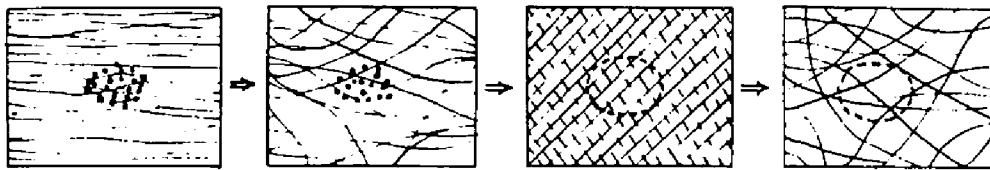
field pit membrane. It is also apparent from observation of the split radial surface between tracheid and ray parenchyma cell (Fig. 63), that microfibrils run along the longitudinal direction of ray cells in the external surface of mature one. Roelofsen (1951) has noted that the orientation of cellulose microfibrils is approximately transverse to the cell axis in the primary wall of parenchyma cells. However, the microfibrillar orientation of the primary wall is parallel to the cell axis in the ray parenchyma cell examined in this study. This observation might be explained that the strong expansion of the cell in its longitudinal direction causes reorientation of microfibrils in the direction.

The inner surface of the differentiating ray parenchyma cell in a more advanced stage, depicts a relatively random orientation of microfibrils which extend from the unpitted region to the area of the future pit membrane exhibiting the plasmodesmatal pores (Fig. 64). In this figure the pit border of the adjacent tracheid wall (arrow) is visible through the interstice of the ruptured pit membrane. The layer of randomly oriented microfibrils appears to be fairly thin, and is thought to be the transitional layer of longitudinally oriented microfibrils to the following apparently crisscrossed layer.

The ray parenchyma cell in the subsequent stage of development shows a lamella of microfibrils oriented in 30° - 40° to the long axis of the cell, being crisscrossed with the underlying lamella (Fig. 65). The microfibrils are deposited throughout the cell in an identical

manner, showing the same orientation in the pit membrane and in the unpitted region (Fig. 66). Plasmodesmatal pores found in the pit membrane of a ray parenchyma cell of the early stage of the crisscrossed lamellae deposition disappear during the following crisscrossed lamellae formation. Interconnection between a ray parenchyma cell and the neighbouring tracheid through plasmodesmata may be occluded in this developmental stage. The deposition of the crisscrossed lamellae was also detected in the ray parenchyma cell of *Pinus densiflora*, being consistent with the finding of the existence of the several lamellae in the pit membrane of the window-like pit of *Pinus sylvestris* (Fengel 1970). Frei et al. (1957) showed that the wall of ray parenchyma cells was closely lamellated and contained microfibrils running in two directions, one steeply spiral and the other in a slow spiral based on the observation of etched sections of *Pinus radiata*. The examination of the present study agrees with their findings of a crisscrossed lamellar structure in the ray parenchyma cell wall.

The innermost surface of a ray parenchyma cell, which has completed its differentiation, depicts the randomly oriented microfibrils which also cover the pit membrane (Fig. 67) as noted by Harada (1965) and other workers (Thomas, Nicholas 1968). The developmental sequence of cell wall formation in the cross-field membrane is represented schematically in Text-Fig. 11.



Text-Fig. 11 Diagrammatic representation of the development of the cross-field pit membrane on the ray parenchyma cell side.

When considering the ultrastructure of the cross-field pit membrane, it is important to take account of the wall organization of a ray parenchyma cell, because the complete wall of the cell constitutes the pit membrane. The literature on this subject is incomplete, being limited to two papers (Wardrop, Dadswell 1952; Harada, Wardrop 1960). Harada et al. (1973) have recently examined the structure of the ray parenchyma cell wall in softwoods systematically. They showed that in a mature ray parenchyma cell from *Chamaecyparis obtusa*, the cell wall consists of following four layers from the external to the inner surface; a layer of longitudinally oriented microfibrils; a thin layer of randomly oriented ones; a layer made up of several lamellae alternating their microfibrillar orientations at 60° - 90° ; and a layer of randomly oriented microfibrils.

This observation supports the results found in the present study using the differentiating cells.

3.1.3. Enzymatic treatment of the pit membrane in cross-field pitting

Bamber (1961) showed that the cross-field pit membrane of softwoods was unlignified through the method of safranin-light green staining. It is well known that the electron density of the pit membrane shows less opacity than that of the unpitted wall in sectional observation of electron microscopy. Balatinecz and Kennedy (1967) found that most ray parenchyma cell walls from hard pines are thin and apparently unlignified in the sapwood adjacent to the cambium.

The cross-field pit membranes, when the radial sections of wood are treated with Toluidine blue-O, show purplish in contrast to the green coloured unpitted walls in the sapwoods of *Cryptomeria japonica* and *Chamaecyparis obtusa*. The pit membranes as well as the unpitted walls of the cell from *Pinus densiflora* show purplish in the outer region of the sapwood. This staining reaction indicates that the cross-field pit membranes are unlignified and contain the non-cellulosic polysaccharides according to Feder and O'Brien (1968). It has been noted that the chemical composition may be identified through the observation of the wood treated by several enzymes (Cowling 1965). As the pit membrane are unlignified, it is expected that treatment with pectinase or hemicellulase should provide some infor-

mation on their chemical nature. Incidentally, the present author (Imamura et al. 1974b) examined the bordered pit membrane which were treated with the enzymes in order to investigate the mechanism of their perforation process. The cross-field pit membranes treated with pectinase or hemicellulase showed an interesting mode of enzyme attack.

It is apparent from Fig. 68 that the outer surface of the cross-field pit membrane in the ray cell side in mature condition is rich in amorphous substances. It has been already reported that the pit membranes are coated with a very smooth layer of materials (Harada et al. 1958; Côté 1958) or with incrusting materials of fine granularity (Thomas, Nicholas 1968). The embedded substances were degraded by hemicellulase, and somewhat randomly oriented microfibrils of the pit membrane then revealed (Fig. 69). The amorphous substances in the pit membrane are assumed to be of hemicellulosic nature from this observation of the pit membrane preferentially degraded by hemicellulase.

Figures 70 and 71 show the surfaces of both tracheid and ray parenchyma cell lumina, respectively, in the cross-field pit membrane after pectinase treatment. These figures reveal the erosion of the central part of the pit membrane by the enzyme action. Fig. 72 shows the sectional observation of the pit membrane degraded by the enzyme (arrows). Nicholas and Thomas (1968), and later Bauch et al. (1970) reported that the torus of the bordered pit membrane which contains an appreciable amount of pectin materials is degraded by pectinase. Hence, the central part of the cross-field pit membrane is similar to the torus of the bordered pit membrane in its high content of pectin

materials.

3.1.4. Pits in other walls of ray parenchyma cells

It has been difficult to prepare the replica of the inner surface of the end wall or the transverse wall of a ray parenchyma cell. However, the inner surface of the transverse wall was observed by employing the replicas of the cross sections of the specimen, and that of the end wall was seen continuing from the radial surface.

The simple pits of the transverse wall of a ray parenchyma cell are shown in Fig. 73. The randomly oriented microfibrils are seen in the innermost surface of the pit membrane which has plasmodesmatal pores. The pit membrane of *Pinus densiflora* is slightly depressed below the level of the unpitted wall; In *Chamaecyparis obtusa* and *Cryptomeria japonica* it is more deeply depressed.

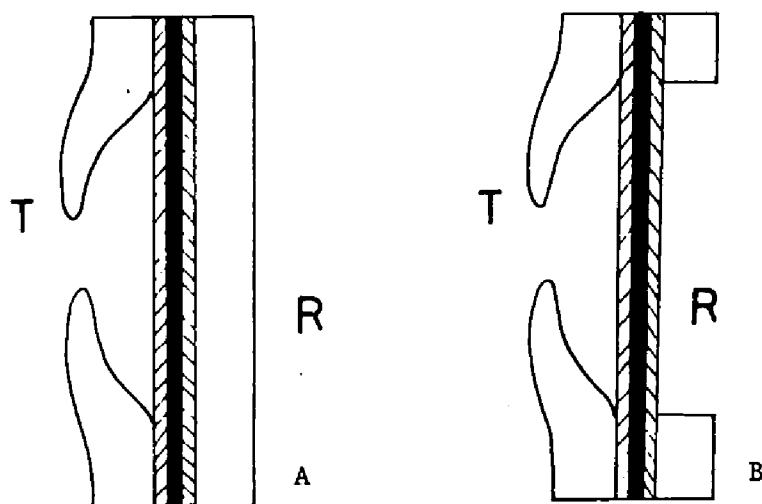
Circular areas with concentrated plasmodesmatal pores were also detected in the corners between the radial and transverse walls, revealing a similar appearance to that of the simple pit in the transverse wall. The pit is assumed to lead to the intercellular space or to be so-called "blind pit" (Preusser et al. 1961). It is also apparent in this case that the microfibrils of the crisscrossed lamellae of a differentiating cell extend to the pit membrane sweeping around the pores (Fig. 74).

Figures 75 and 76 show the inner surfaces of the end walls of a ray parenchyma cells in the mature and differentiating condition, respectively. The end wall with dispersed pores was commonly observed

in three species used in this study, as shown in sectional view by Côté and Day (1968). From this mode of pit structure, it is reasonable to assume that the end wall has been observed as the smooth wall without nodular structure (Panshin, de Zeeuw 1964).

3.2. Development of the pits of thick-walled ray parenchyma cells

Harada (1964) showed a representation of the structure of the cross-field pit membrane which is composed of the primary wall of the tracheid and the complete wall of the ray parenchyma cell (Text-Fig. 12A). The assumption has been confirmed by following studies (Thomas, Nicholas 1968; Fengel 1970). However, the textbook of wood structure has generally shown the representation that the pit aperture of the ray parenchyma lumen side is open (Text-Fig. 12B). This structure was recognized in the half-bordered pit pair of the hardwood.



Text-Fig. 12 Diagrammatic representation of a half-bordered pit pair of softwood. T; tracheid, R; ray parenchyma cell (Harada 1964).

Incidentally, the examinations of the pit structure have been done using mainly the species which are constituted of the thin-walled ray parenchyma cells. It is sometimes noted that some species, however, have the thick-walled ray parenchyma cells, showing the conspicuous nodules in the radial sections. It is predicted that the ray parenchyma cell of the latter type exhibits the pit structure as shown in Text-Fig. 12B. *Abies firma* which is constituted of the thick-walled ray cell as *Picea*, *Pseudotsuga*, *Tsuga* and so on was employed.

3.2.1. Pit membrane leading to the tracheid

In the inner surface of the ray parenchyma cell in the very early stage of development, microfibrils are deposited in parallel to the long axis of the ray cell. The microfibrils run in relatively random in the area with plasmodesmatal pores, which is assumed to develop into the cross-field pit membrane.

The lamellae being crisscrossed each other were recognized in the cell in a little more advanced stage (Fig. 77). The microfibrils exhibit the same appearance throughout the pit membrane and the unpitted wall region. The layer of the crisscrossed lamellae was also found in the thin-walled ray parenchyma cell, and is assumed to be specific to the parenchymatous cell walls (Imamura et al. 1974c).

The layer of the microfibrils in random orientation is deposited, subsequent to the formation of the layers described above (Fig. 78). The circular depression indicates the pit membrane showing no plasmodesmatal pores. The appearance is very similar to that of the inner

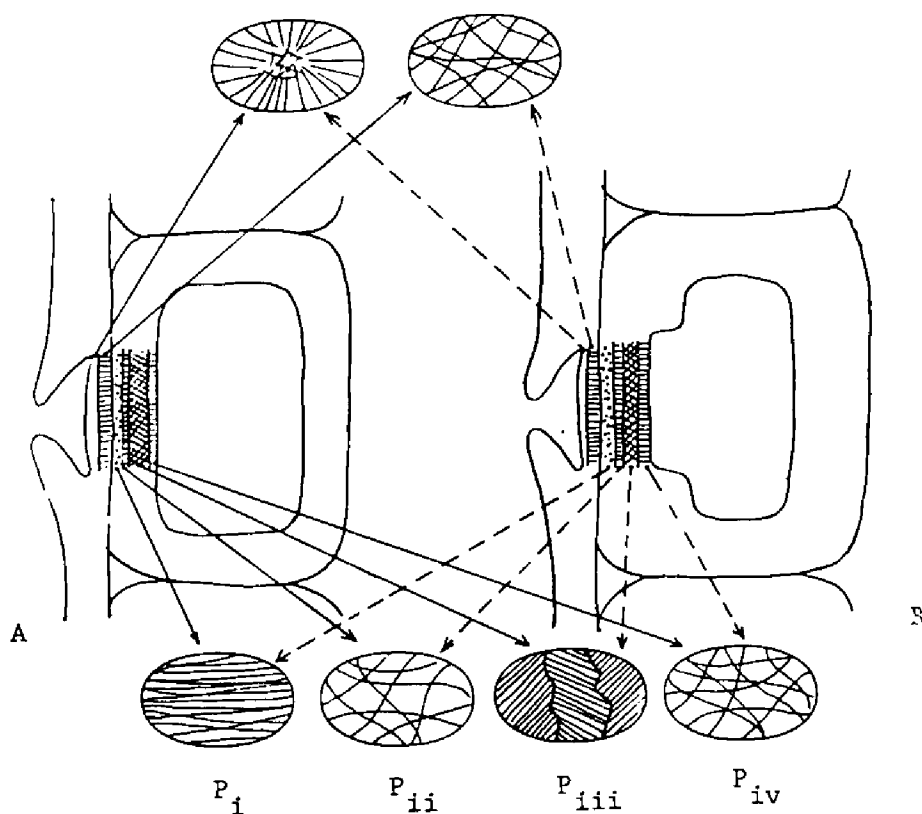
surface of the thin-walled ray parenchyma cell in mature condition (cf. Fig. 67). The thick-walled ray parenchyma cell does not complete the cell wall formation by this stage, but shows the continuous formation of the following layer (S-layer).

The ray cell in the next developmental stage shows the microfibrils sweeping around the pit membrane (Fig. 79). The microfibrils are preferentially deposited at first in the periphery of the pit membrane, and then in the unpitted region. The pit membrane remains intact in the random orientation.

Figure 80 shows the inner surface of the ray cell in the middle stage of the formation of the S-layer. A detail examination of the microfibrillar orientation in the pit region establishes that the appearance of the layer is similar to that of the secondary wall of the tracheid, whereas the pit border is not developed in this case. This is supposed to be resulted from that the microfibrils could not be deposited in the proceeding manner of microlamellae which is explained in the tracheid wall.

Figures 81 and 82 show the inner surface of the mature ray parenchyma cell and the sectional appearance of the cross-field pit membrane, respectively. It is apparent that the pit in the ray cell side exhibits the typical simple pit structure. Very recently, Fujikawa (1974) has proposed a new concept similar to that presented above regarding the cell wall organization.

Text-Fig. 13 shows a schematic drawing of a generalized pattern of wall organization based on the present observation on the cell wall



Text-Fig. 13 Diagrammatic representation of the cell wall layers of the cross-field pit membrane of the ray cell. A; thin-walled ray cell, B; thick-walled ray cell.

structure of the cross-field pit membrane of ray parenchyma cells.

The P_i represents the wall layer in which microfibrils run in parallel to the cell axis, the P_{ii} and P_{iv} show the layers of randomly oriented microfibrils and the P_{iii} does the layer composed of the lamellae alternating their orientation in crisscrossed. These layers are generally found in the thin and thick-walled ray cells, making up the pit membrane. In the thick-walled ray cell, however, the secondary layer is added in the unpitted region.

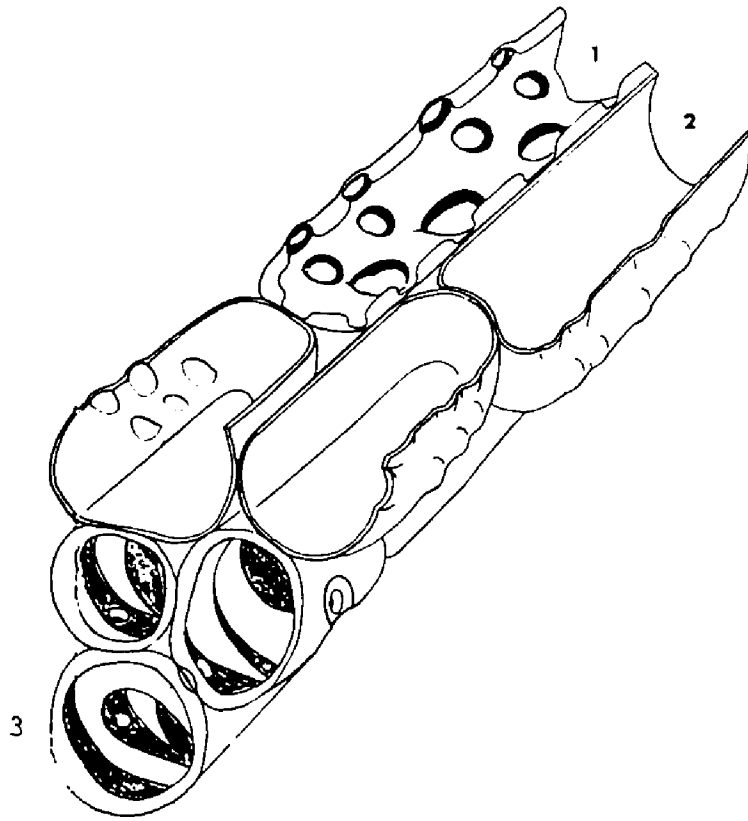
The assumption that the S-layer is laid down after the deposition of the layers in the thin-walled ray parenchyma cell is more plausible, in considering the finding of the thick-walled ray cells in pine. In hard pines with pinoid pits, including the southern pines, numbers of thick-walled cells increase at sapwood-heartwood boundary (Balatinecz, Kennedy 1967). Côté and Day (1967) have also noted that some ray cells in heartwood of table mountain pine are thick-walled and show the half-bordered pit pair as represented in Fig. 82. Moreover Howard and Manwiller (1969) presented the cell wall structure of this kind of cells in Text-Fig. 14. They noted that thin and thick-walled forms may be found in the same ray, and that the thick-walled cells locate near the ray margins, usually adjacent to ray tracheids.

The result described above suggests that the cell wall of the thick-walled ray cell is composed of the wall of the thin-walled ray cell and the additioned S-layer.

3.2.2. Interparenchymatous pit membrane

In the transverse and end walls between two parenchyma cells, voids of wall could be detected. Fig. 83 shows the transverse wall in the initial stage of the S-layer formation. Microfibrils run around the pit membrane. In different with the cross-field pit membrane, the simple pit membrane exhibits plasmodesmatal pores. The S-layer is deposited in the unpitted region in the same manner as observed in the radial wall.

The thin-walled ray parenchyma cell exhibits the end wall



Text-Fig. 14 Diagrammatic representation of the sectioned ray parenchyma. 1; thick-walled parenchyma with simple pitting, 2; thin-walled parenchyma, 3; dentate ray tracheid (Howard, Manwiller 1969).

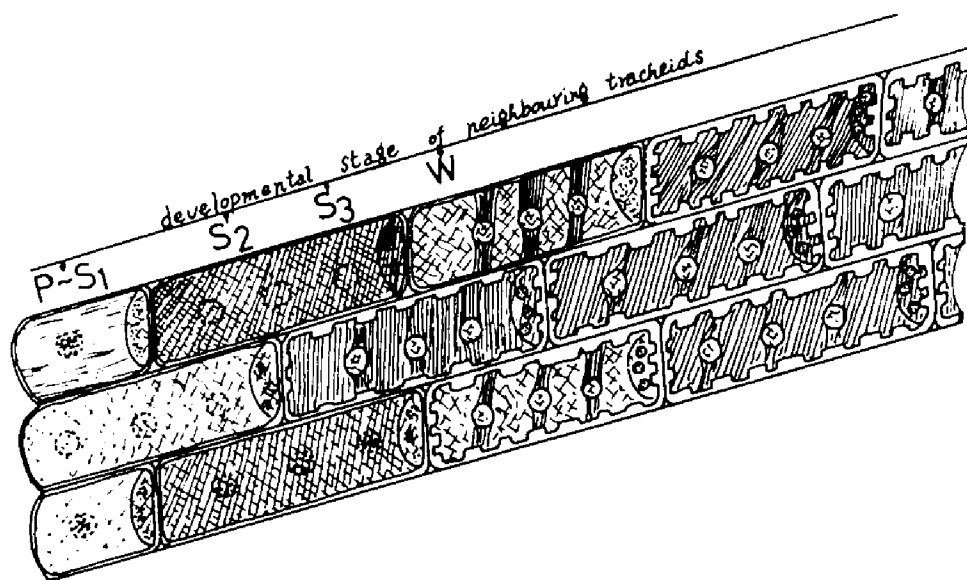
scattered with plasmodesmatal pores, whereas the typical simple pits could be detected in the end wall of the thick-walled ray cell. The simple pits in the stage of the S-layer deposition is shown in Fig.

84.

3.2.3. Developmental sequence of the wall of the ray parenchyma cells

In a radial file of the differentiating xylem, developmental

sequence of the ray parenchyma cells is hardly coincident with that of the neighbouring tracheids. Moreover, a ray cell is adjacent to three to four tracheids in different developmental stages. The development of ray cells is generally delayed to that of the neighbouring tracheids in the formation stage of the cell wall. The samples collected in May have no mature ray cell, though the tracheid cell walls have been completely developed. Thus it is necessary to collect the samples in the latewood formation season for observation of the ray cells in the different stages of all cell wall formation. Text-Fig. 15 shows a diagrammatic representation of the differentiating



Text-Fig. 15 Diagrammatic representation of cell wall formation and pit development of thick-walled ray parenchyma cells.

ray parenchyma cells in association with neighbouring tracheids.

Interconnection between a ray cell and a tracheid through plasmodesmatal pores of a cross-field pit membrane was observed to be occluded in the stage of the crisscrossed lamellae deposition. In this stage, the neighbouring tracheid has completed its wall formation and is apparent to be the non-living cell. It is supposed that the pit membrane in the P_{iii} formation (Fig. 78) possesses a protecting function for the living protoplast against the adjoining dead cell. In hardwood ray cells, the additioned layer which is observed at the vessel-parenchyma pit membrane is considered to have the protecting function and is termed the "protective layer" (Schmid 1965).

3.3. Development of the pits of axial parenchyma cells

Axial or longitudinal parenchyma cells are found in various rate and distribution pattern in the growth ring. They are generally short and thin-walled elements, occurring in strands along the grain (Kollman, Côté 1965).

In *Crptomeria japonica* and *Chamaecyparis obtusa*, the axial parenchyma cell is called the resin cell because of containing oil droplets.

In pines, axial parenchyma cells partially surround most of the longitudinal resin canal. The parenchyma cell lies in strand just outside the sheath of epithelial cells. When more than one layer of cells is present, cells of the innermost strand are slightly longer than the epithelial cells; those of the outermost layer are $1\frac{1}{2}$ to

three times as long (Howard, Manwiller 1969). The two kinds of cells are identical with respect to lamination, pitting, intercellular perforations and elevated cross walls (Kibblewhite, Thompson 1973).

Resin canal or resin cells appear almost in earlywood-latewood boundary or in latewood. Replicas of radial sections of the samples in the latewood formation season show tracheids in various stages in which each layer is undergoing formation, but do only a small number of axial parenchyma cells.

3.3.1. Pit membrane between tracheid and axial parenchyma cell

Pits of the tracheids adjacent to the axial parenchyma cell are similar in structure to cross-field pits of the thin-walled ray parenchyma cell. These pits, however, are arranged along the longitudinal direction of the cell.

In the pit membrane, radiating structure of microfibrils is recognized as shown in Fig. 85. The observation of the inner surface of the tracheid in the developmental stage makes it clear that the radiating structure is constructed in the formation stage of the primary wall. The structure and developmental sequence of the pit are assumed to be same with those of the cross-field pits.

Figure 86 shows the inner surface of a resin cell in the very early stage of the development. The wall adjacent to the tracheid is composed of microfibrils which are almost transverse to the cell axis, but in relatively interwoven. The area where abundant plasmodesmatal pores are detected is assumed to be the pit membrane interconnecting

with the tracheid.

In a more advanced stage, the resin cell shows the crisscrossed lamellae deposition (Fig. 87). The microfibrils of the layer run in the same manner both in the unpitted region and in the pit membrane. Randomly oriented microfibrils could be recognized in the innermost surface of the cell (Fig. 88). The circular depressed area is assumed to be a pit membrane, showing the similar appearance with the cross-field pit membrane in the thin-walled ray parenchyma cell.

The sectional observation confirms the result that the pit membrane consists of the primary wall of the tracheid and the complete wall of the axial parenchyma cell.

3.3.2. Interparenchymatous pit membrane

The resin cell of the two species is surrounded only by tracheids, and hardly adjacent to other parenchyma cell. However, the axial parenchyma cells in the resin canal region of *Pinus densiflora* locate in group around an epitherial cell. Interparenchymatous pit membranes were examined in the lateral walls between two parenchyma cells, viz. axial parenchyma cell-axial parenchyma cell and axial parenchyma cell-ray parenchyma cell.

Figure 89 shows the inner surface of the axial parenchyma cell in the formation stage of the crisscrossed lamellae. The area with abundant pores is assumed to be the pit membrane leading to the other axial parenchyma cell, which is supposed to be equal to the primary pit field. Microfibrils continue to the pit membrane from the unpitted

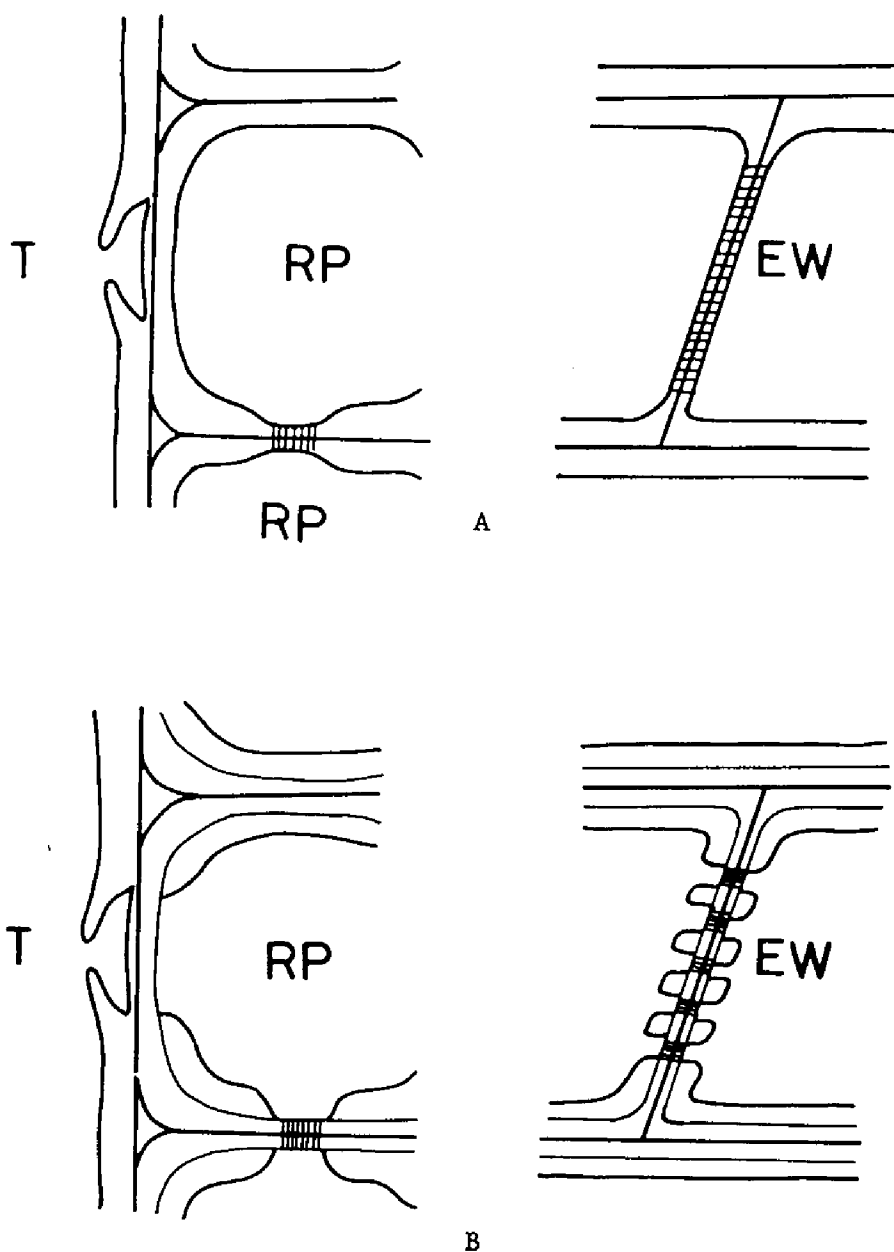
region, curving around the plasmodesmatal pores. The plasmodesmatal pores can be recognized in the mature condition. The appearance and developmental sequence of the pit membrane are very similar to those in the transverse walls of thin-walled ray parenchyma cells.

The pit structure adjacent to the ray parenchyma cell is also interpreted to be same as described above (Fig. 90).

3.4. Summary

The development and ultrastructure of pits of the thin and thick-walled ray parenchyma cells and axial parenchyma cells of softwoods were examined through observations of the inner surfaces of the differentiating parenchyma cells and neighbouring tracheids.

Thin-walled ray parenchyma cells of *Pinus densiflora*, *Cryptomeria japonica* and *Chamaecyparis obtusa* were studied at first. Radiating microfibrils were recognized on the tracheid lumen surface of the cross-field pit membrane in the later stage of primary wall formation. It was revealed through enzyme treatment that the cross-field pit membrane in mature condition is embedded in amorphous substances of hemicellulosic nature, and the center of the membrane is especially rich in pectic materials. On the thin-walled ray parenchyma cell side, successive layers are deposited, including a layer of microfibril orientation in the longitudinal direction of the cell, a layer of randomly oriented microfibrils, several lamellae alternating their microfibril orientation in a crisscrossed pattern and the innermost layer of randomly oriented microfibrils. Simple pits, which can not



Text-Fig. 16 Diagrammatic representation of the pit structure of parenchyma cells. A; thin-walled ray parenchyma cell, B; thick-walled ray parenchyma cell. T; tracheid, RP; ray parenchyma cell, EW; end wall.

be distinguished from primary pit field, were noted as circular depressions with abundant plasmodesmatal pores in the transverse wall between two parenchyma cells and the cell corner opposite to the intercellular space. In the end wall, however, the pores are dispersed (See Text-Fig. 16A).

In the thick-walled ray parenchyma cell of *Abies firma*, the wall layer (S-layer) similar to the secondary wall of the tracheid was detected to be laid down following the deposition of the complete wall layers found in the thin-walled ray parenchyma cell. Microfibrils of the S-layer, however, are deposited keeping away from the pit membrane, resulting in the formation of the typical simple pit. Plasmodesmatal pores were observed only in the interparenchymatous pit membrane (See Fig. 16B).

The development of the pit of the axial parenchyma cells was examined of *Cryptomeria japonica* and *Chamaecyparis obtusa* (resin cell), and of *Pinus densiflora* (resin canal complex). The developmental sequences of the pit are assumed to be essentially similar to those of the thin-walled ray parenchyma cell. The outermost layer, however, shows the microfibrillar orientation perpendicular to the cell axis.

Conclusion

The development and ultrastructure of pits of coniferous xylem were studied through observation of the inner surface of the differentiating cell. This observation method was successful using plasmolysis treatment and freeze-drying technique. New evidences were obtained regarding the pit development of the tracheid and parenchyma cell.

The process of the formation of the bordered pit membrane was examined in relation to the tracheid wall development. The microfibrillar structure of the bordered pit membrane is constructed in the formation stage of the primary wall, showing the same appearance as that of the mature one. The pit membrane, however, is fully embedded in the amorphous substances throughout the differentiation zone, and is perforated after the cell wall formation is completed. It is assumed that the degradation of the embedding substances is caused by the enzyme such as hemicellulase and that the torus is resistant to the enzyme action by abundance of pectin materials.

It is recognized that the pit border is developed by formation of the S_1 -layer in the tracheid lumen side and simultaneous deposition of initial border thickening in the pit chamber side. In the S_1 -layer formation, microlamellae which consist of microfibrils lying parallel in the constant distance, are successively deposited and curve around the pit aperture resulting in the pit border elongation. The S_2 -layer as well as the S_1 -layer contributes to the pit border development in the latewood.

The development of pits of parenchyma cells was also examined.

The cross-field pit membrane on the tracheid side shows the radiating structure of microfibrils with the similar appearance to that of the bordered pit membrane. The pit membrane of the ray parenchyma cell consists of the layer of microfibrils oriented in parallel to the ray axis (P_i), the layer of randomly oriented microfibrils (P_{ii}), several lamellae alternating their orientation in crisscrossed (P_{iii}) and the innermost layer of randomly oriented microfibrils (P_{iv}). In the thick-walled ray parenchyma cell, the P_i - P_{iv} are commonly observed in the pit membrane, while the addition layer (S-layer) is deposited in the unpitted region. Plasmodesmatal pores are only recognized in the inter-parenchymatous pit membrane even in the mature condition.

It is assumed that examination of the inner surface of the differentiating cell is a useful method to study the cell wall structure. In investigation on the cell wall in differentiation, attention should be paid for interpretation of the sequence and appearance of the cell wall development. In studying the differentiating cells which do not form a sequential radial file, such as parenchyma cells, the developmental stage should be proven by comparing the appearance of the sample each other, and by referring with the cell wall organization of the neighbouring elements. The condition of the cell wall formation is supposed to be controlled by the physiological activity of the tree, and may sequentially change in 24 hours of a day (Bobák, Necěsaný 1967). Thus, it appears necessary to collect the sample in the different time.

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Explanation of figures

Figure

1. Direct carbon replica of the radial inner surface of a differentiating tracheid showing the adhered cytoplasm on the S_1 -layer. *Pinus densiflora*. In this and the following figures, the longitudinal cell axis is perpendicular to the direction of scale line except the figures, in which it is shown by FA (fiber axis).
2. Direct carbon replica of the inner surface of a differentiating tracheid showing the microfibrillar orientation of the outer surface of the primary wall. *Pinus densiflora*.
3. Similar to Fig. 2, but showing the microfibrillar orientation of the inner surface of the primary wall.
4. Cross section of the differentiating tracheid treated with hypertonic agent, and then stained with $KMnO_4$.
5. Direct carbon replica of the inner surface of a differentiating tracheid in the formation stage of the S_2 -layer. The sample was treated with hypertonic agent and then freeze-dried. *Pinus densiflora*.
6. Direct carbon replica of the inner surface of a differentiating tracheid in the S_1 -layer formation. Note that the material is attaching to the wall. *Pinus densiflora*.
7. Direct carbon replica of the inner surface of a differentiating tracheid in the formation stage of the S_2 -layer. Note the granules adhering to the cell wall. *Pinus densiflora*.
8. Direct carbon replica of a bordered pit membrane, prepared through the solvent-exchange drying. *Pinus densiflora*.
9. Direct carbon replica of a bordered pit membrane, prepared through the freeze-drying method. *Pinus densiflora*.
10. Direct carbon replica of the inner surface of a developing earlywood tracheid treated with Jeffrey's solution, showing a primary pit field. PD: plasmodesmata
11. Direct carbon replica of the inner surface of a developing earlywood tracheid showing the initiation of the formation of radiating microfibrils in the margo of the pit membrane (arrow).

12. Direct carbon replica of the inner surface of a developing earlywood tracheid, showing the fully developed bordered pit membrane and the initiation of pit border formation (arrow).
13. Similar to Fig. 12, but showing a bordered pit membrane disclosed by treatment with Jeffrey's solution. Note the pit membrane having the same microfibrillar structure as that of the mature one.
14. Direct carbon replica of the split radial surface of a developing earlywood tracheid showing a bordered pit membrane embedded in matrix substances.
15. Similar to Fig. 14, but showing a developing pit membrane disclosed partially by the solvent-exchange treatment.
16. Similar to Fig. 15, but showing the presence of warts on the outer surface of the pit border which is seen through a broken pit membrane.
17. Direct carbon replica of a split radial surface of a mature earlywood tracheid showing a perforated pit membrane.
18. Direct carbon replica of a split radial surface of a mature earlywood tracheid from *Cryptomeria japonica*. (reversal)
19. Similar to Fig. 18, but showing a bordered pit membrane of a developing earlywood tracheid from *Cryptomeria japonica*. (reversal)
20. Similar to Fig. 19, but showing the profiling of cellulose microfibrils disclosed by treatment with Jeffrey's solution. *Chamaecyparis obtusa*. (reversal)
21. Direct carbon replica of the inner surface of a developing earlywood tracheid through chemical treatment. *Chamaecyparis obtusa*. (reversal)
22. Direct carbon replica of a differentiating bordered pit membrane between the ray and longitudinal tracheid. *Pinus densiflora*.
23. Similar to Fig. 22, but showing a bordered pit membrane in the mature condition.
24. Direct carbon replica of the inner surface of a developing earlywood tracheid in the primary wall formation, showing the initiation of the radiating microfibril deposition in the margo. The specimen was treated with Jeffrey's solution. *Pinus densiflora*.

25. Similar to Fig. 24, but showing a more developed pit membrane visualized through treatment with 24% KOH. *Chamaecyparis obtusa*.
26. Direct carbon replica of the split radial surface of a developing earlywood tracheid from *Pinus densiflora* treated with 5% KOH. (reversal)
27. Similar to Fig. 26, but showing a fully disclosed pit membrane by treatment with 24% KOH. (reversal)
28. Direct carbon replica of a bordered pit membrane from a differentiating tracheid after boiling treatment, showing the interstices in the margo. *Chamaecyparis obtusa*. (reversal)
29. Direct carbon replica of an earlywood bordered pit membrane in the last stage of its development. *Cryptomeria japonica*. (reversal)
30. Similar to Fig. 29, but showing the pit membrane in the very late stage of the degradation process. *Pinus densiflora*. (reversal)
31. Similar to Figs. 29 and 30, but showing the almost perforated pit membrane. *Pinus densiflora*. (reversal)
32. Direct carbon replica of the split radial surface of a differentiating earlywood tracheid, showing a bordered pit membrane degraded slightly in the margo through treatment with hemicellulase for 3 days. *Pinus densiflora*.
33. Similar to Fig. 32, but showing a more advanced stage of the degradation. *Pinus densiflora*.
34. Similar to Fig. 32 and 33, but showing an almost completely perforated pit membrane from the sample treated with the enzyme for 5 days. Note the small amount of the embedding substances attaching to the margo microfibrils. *Pinus densiflora*.
35. Direct carbon replica of a pit membrane from a differentiating tracheid treated with pectinase for 3 days. Note the degradation in the future torus region. *Pinus densiflora*.
36. Direct carbon replica of a differentiating bordered pit membrane treated with hemicellulase for 3 days, showing the almost perforated margo. *Cryptomeria japonica*.
37. Direct carbon replica of a split radial surface of a mature latewood tracheid from *Pinus densiflora*.

38. Similar to Fig. 37, but showing the improved profiling of cellulose microfibrils through treatment with 24% KOH. (reversal)
39. Similar to Fig. 37, but showing a margo without any incrustation of amorphous substances through the hemicellulase treatment.
40. Direct carbon replica of a bordered pit membrane from a mature tracheid of *Abies firma*, showing its torus extension.
41. Direct carbon replica of a differentiating bordered pit membrane treated with hemicellulase, showing a margo without any incrustation. *Abies firma*.
42. Direct carbon replica of the inner surface of a differentiating vessel element, showing the perforation partition embedded in the amorphous substances. *Cercidiphyllum japonicum*.
43. Similar to Fig. 42, but showing the dense structure of microfibrils in the perforation partition through the treatment of hemicellulase.
44. Direct carbon replica of the differentiating perforation partition in the initial stage of the degradation.
45. Similar to Fig. 44, but showing the more developed stage of the degradation.
46. Similar to Fig. 12, but showing the developing S_1 -layer (S_1), a pit membrane (PM) and the outer surface of the developing pit border of the adjacent tracheid seen through the interstice of the broken pit membrane (BT). *Pinus densiflora*.
47. Direct carbon replica of the inner surface of a differentiating earlywood tracheid, showing the deposition of microfibrils in the S_1 -layer around the developing pit aperture.
48. High magnification of Fig. 46. Note the microfibrils sweeping around the pit aperture and some of them extending beyond the tip of the border.
49. Direct carbon replica of the inner surface of a differentiating tracheid in the formation stage of the S_1 -layer. Note the deposition of microlamellae. *Pinus densiflora*.
50. Direct carbon replica of the inner surface of the differentiating tracheid, showing the progressive development of successive microlamellae, (1) - (2) - (3). *Pinus densiflora*.

51. Ultra-thin tangential section of the developing earlywood tracheids showing the sectional view of the bordered pit pair. Note the scale-like texture of the pit border visualized by staining with KMnO_4 .
52. A model showing the deposition of microfibrils of the S_1 -layer in the pit border region.
53. Direct carbon replica of the inner surface of the differentiating tracheid, showing the S_2 -layer orientation in the pit border region with the aid of specimen inclining apparatus. *Pinus densiflora*.
54. Similar to Fig. 53, but showing the deposition of the microlamellae in the pit border. *Pinus densiflora*.
55. Ultra-thin cross section of the developing earlywood tracheids stained with KMnO_4 , showing the sectional appearance of the pit border. *Pinus densiflora*.
56. Ultra-thin radial section of the developing earlywood tracheid stained with KMnO_4 . *Pinus densiflora*.
57. Direct carbon replica of the inner surface of a mature latewood tracheid, showing the lenticular pit aperture.
58. Direct carbon replica of the inner surface of a differentiating latewood tracheid in the S_2 -layer formation. *Pinus densiflora*.
59. Similar to Fig. 58, but showing the stream line orientation of microfibrils around the pit border and supplementary deposition of concentrically oriented microfibrils around the aperture. *Pinus densiflora*.
60. Direct carbon replica of a cross-field pit membrane as viewed from the tracheid lumen, showing the improved profiling of the radiating microfibril structure following treatment with Jeffrey's solution. *Chamaecyparis obtusa*. In this and the following figures, the ray axis is parallel to the direction of scale line except the figures, in which it is shown by RA (ray axis).
61. Direct carbon replica of the inner surface of a differentiating tracheid in primary wall formation, showing the full development of the microfibrillar structure of the cross-field pit membrane and the initiation of pit border development (arrows). *Chamaecyparis obtusa*.
62. Direct carbon replica of the inner surface of a differentiating

ray parenchyma cell in a very early developmental stage.
Chamaecyparis obtusa.

63. Direct carbon replica of the split radial surface of the mature sample treated with Jeffrey's solution. The cross-field pit membrane of the tracheid side is peeled back, allowing the observation of the external surface of the ray parenchyma cell.
Cryptomeria japonica.
64. Similar to Fig. 63, but showing the random orientation of microfibrils of the cell in a more advanced stage. The arrow indicates the outer surface of the pit border of the adjacent tracheid.
65. Direct carbon replica of inner surface of a differentiating ray parenchyma cell in the criss-crossed lamellae deposition. The long arrow shows the direction of the newly deposited lamella, and the short one indicates that of the underlying lamella.
66. Similar to Fig. 65, but showing the cross-field pit membrane.
67. Direct carbon replica of the inner surface of a mature ray parenchyma cell, showing the random orientation of microfibrils in the cross-field pit membrane. *Chamaecyparis obtusa*.
68. Direct carbon replica of the split radial surface of ray cell from *Chamaecyparis obtusa*, showing the incrustation of amorphous substances on the outer surface of the pit membrane in the ray cell side.
69. Similar to Fig. 68, but showing the improved profiling of microfibrils through the hemicellulase treatment.
70. Direct carbon replica of the cross-field pit membrane from *Chamaecyparis obtusa* eroded by pectinase action, as viewed from the tracheid lumen side.
71. Similar to Fig. 70, but the eroded pit membrane as viewed from the ray cell lumen.
72. Ultra-thin cross section of a ray parenchyma cell shadowed with Pt-Pd, showing the erosion of the cross-field pit membrane through pectinase treatment. *Chamaecyparis obtusa*.
73. Direct carbon replica of the inner surface of the transverse wall of a mature ray parenchyma cell from *Pinus densiflora*, showing the surface view of the simple pits.

74. Direct carbon replica of the pit leading to an intercellular canal, as viewed from the ray cell (arrows). Note the deposition of the crisscrossed lamellae in the radial wall (below the pit in the photo). *Chamaecyparis obtusa*.
75. Direct carbon replica of the inner surface of an end wall in the mature condition. *Cryptomeria japonica*.
76. Similar to Fig. 75, but showing an end wall in the differentiating stage of the criss-crossed lamellae deposition. The surface is observed continuing from the radial wall (leftside of the photo). *Cryptomeria japonica*.
77. Direct carbon replica of the inner surface of a differentiating thick-walled ray parenchyma cell, in the formation stage of the crisscrossed lamellae. *Abies firma*.
78. Similar to Fig. 77, but showing the random orientation of microfibrils in the pit membrane (circular depression) and the unpitted wall.
79. Similar to Fig. 78, but showing the initial stage of deposition of the secondary wall layer (S-layer) PM: pit membrane of the cross-field pit.
80. Similar to Fig. 79, but showing the more advanced stage of the S-layer formation.
81. Direct carbon replica of the inner radial surface of a thick-walled ray parenchyma cell in the mature condition.
82. Ultra-thin tangential section of a ray parenchyma cell shadowed with Pt-Pd. *Abies firma*. (reversal)
83. Direct carbon replica of the inner surface of a differentiating thick-walled ray parenchyma cell, showing the developing simple pit in the transverse wall.
84. Similar to Fig. 83, but showing the developing simple pits in the end wall.
85. Direct carbon replica of the inner surface of a tracheid adjacent to the axial parenchyma cell. *Pinus densiflora*.
86. Direct carbon replica of the inner surface of a differentiating resin cell of *Cryptomeria japonica*. Note the transversely oriented microfibrils and the developing pit membrane (arrows).
87. Direct carbon replica of the inner surface of a differentiating

resin cell, showing the deposition of the crisscrossed lamellae.
Chamaecyparis obtusa.

88. Direct carbon replica of the inner surface of a mature resin cell, showing the pit membrane leading to the neighbouring tracheid. *Cryptomeria japonica*.
89. Direct carbon replica of the inner surface of a differentiating axial parenchyma cell in the formation stage of the crisscrossed lamellae. Note the pit membrane leading to the other axial parenchyma cell. *Pinus densiflora*.
90. Similar to Fig. 89, but showing the pit membrane leading to the ray parenchyma cell. *Pinus densiflora*.

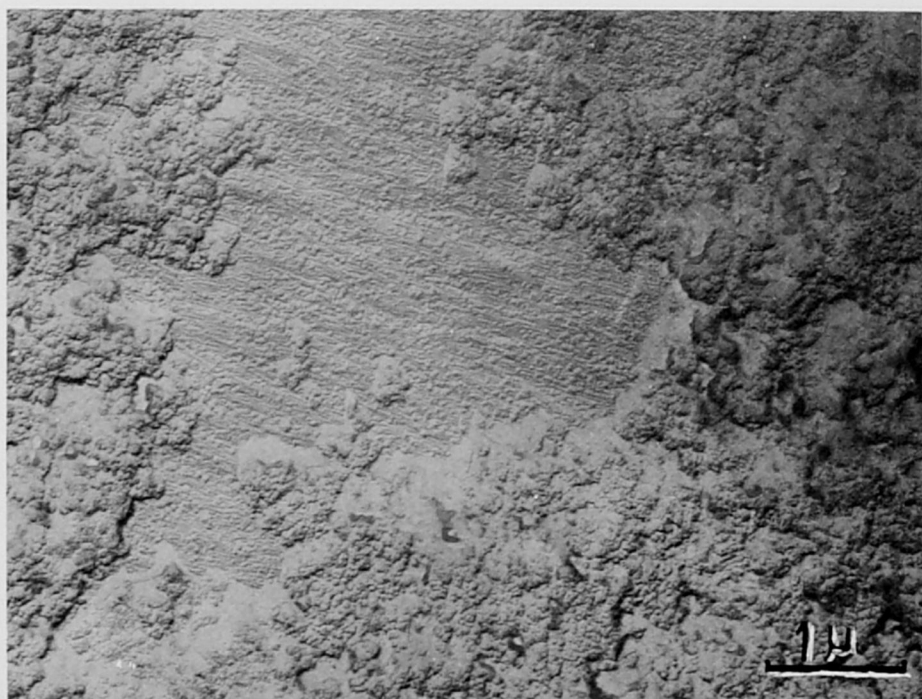


Fig. 1



Fig. 2

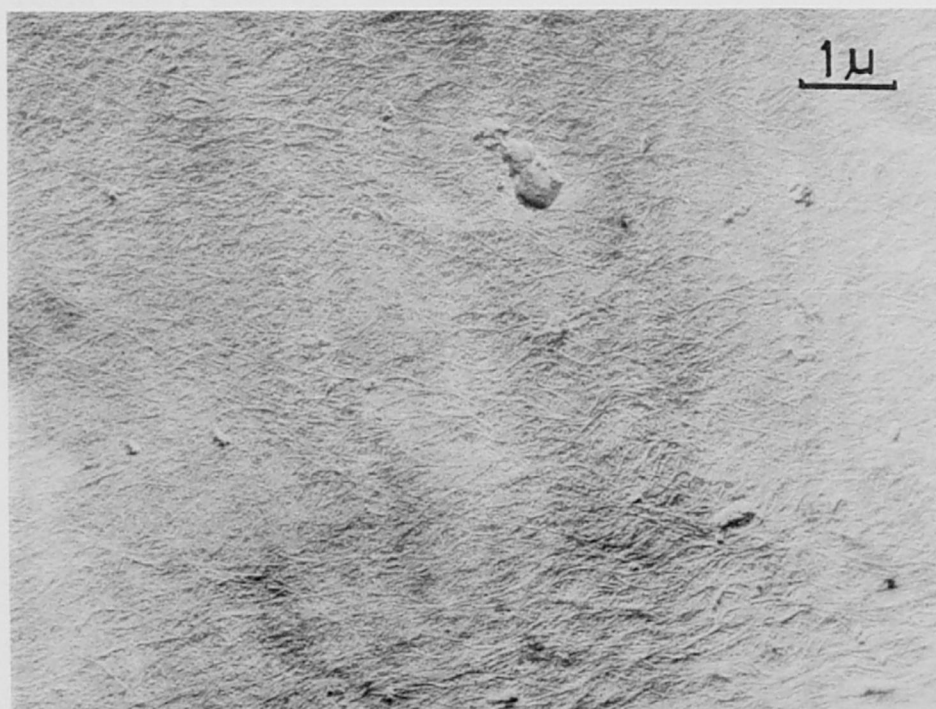


Fig. 3

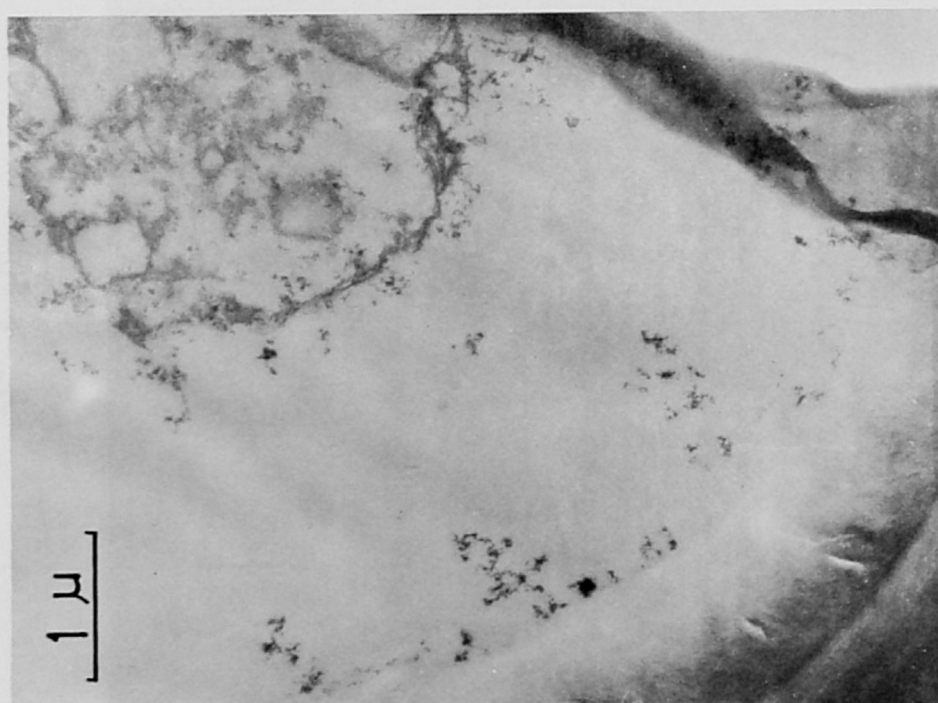


Fig. 4



Fig. 5

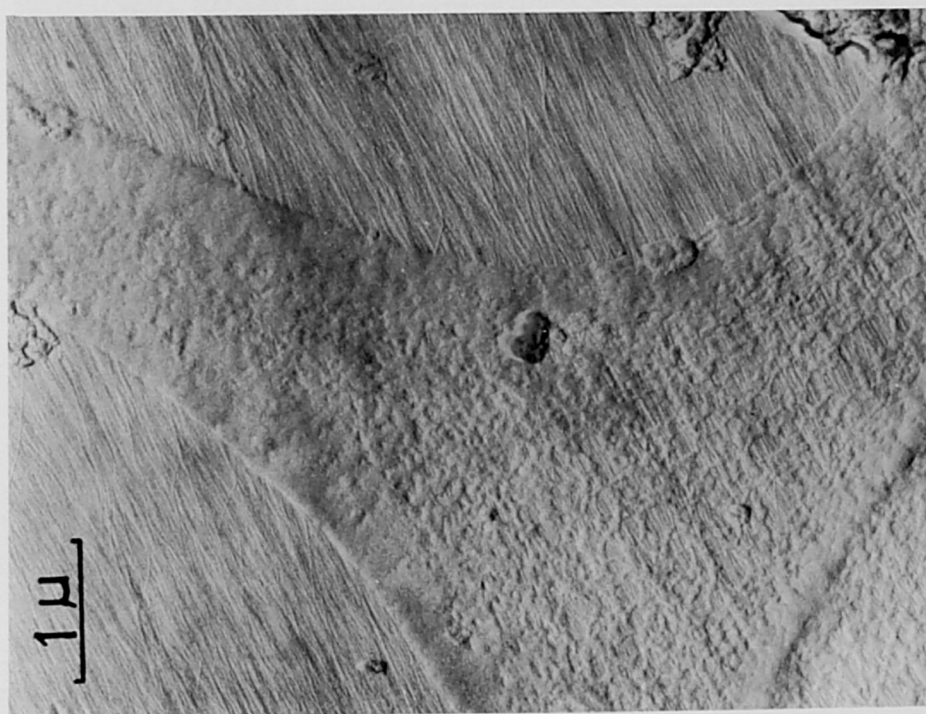


Fig. 6

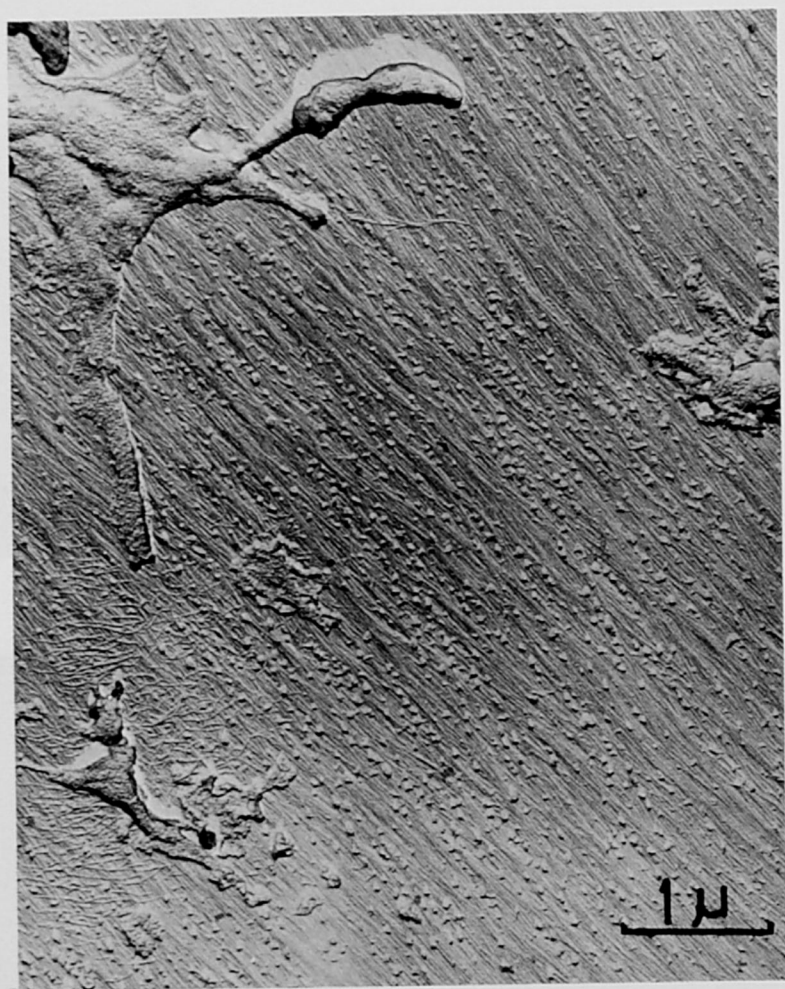


Fig. 7



Fig. 8

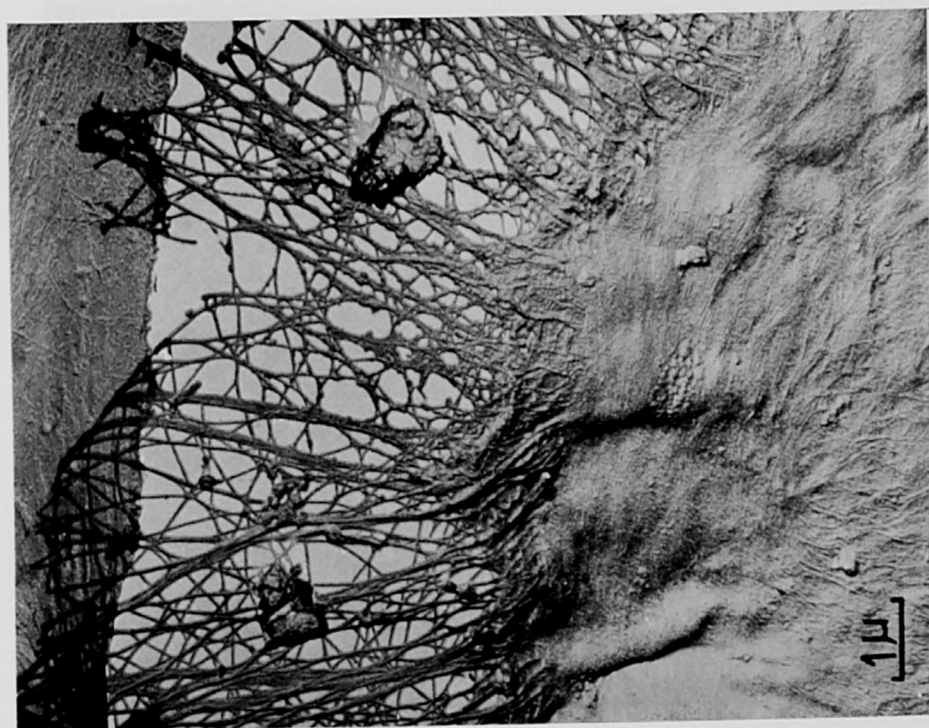


Fig. 9

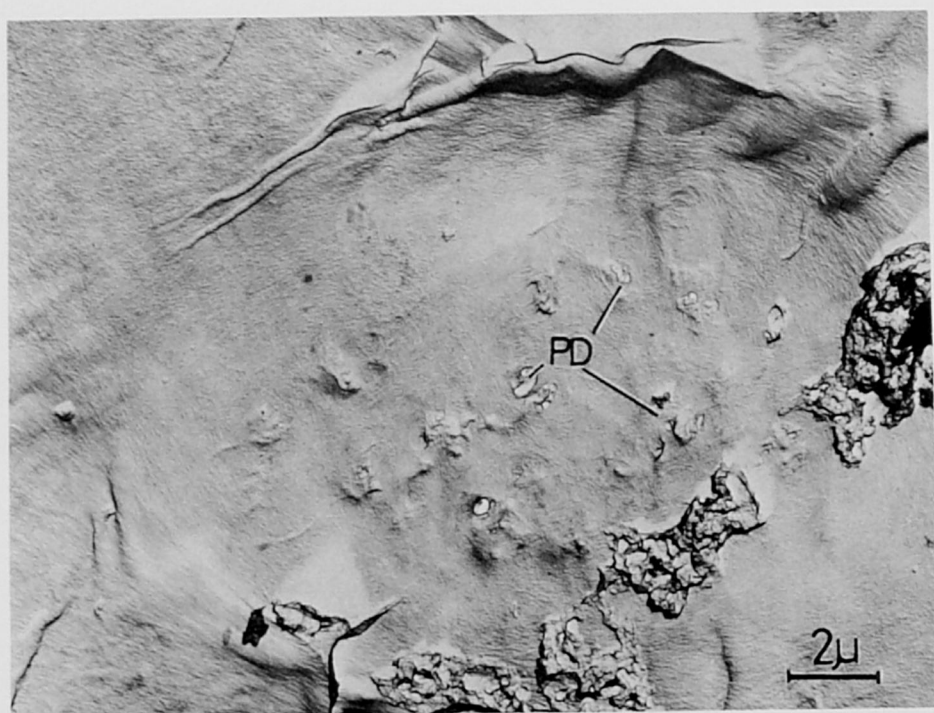


Fig. 10

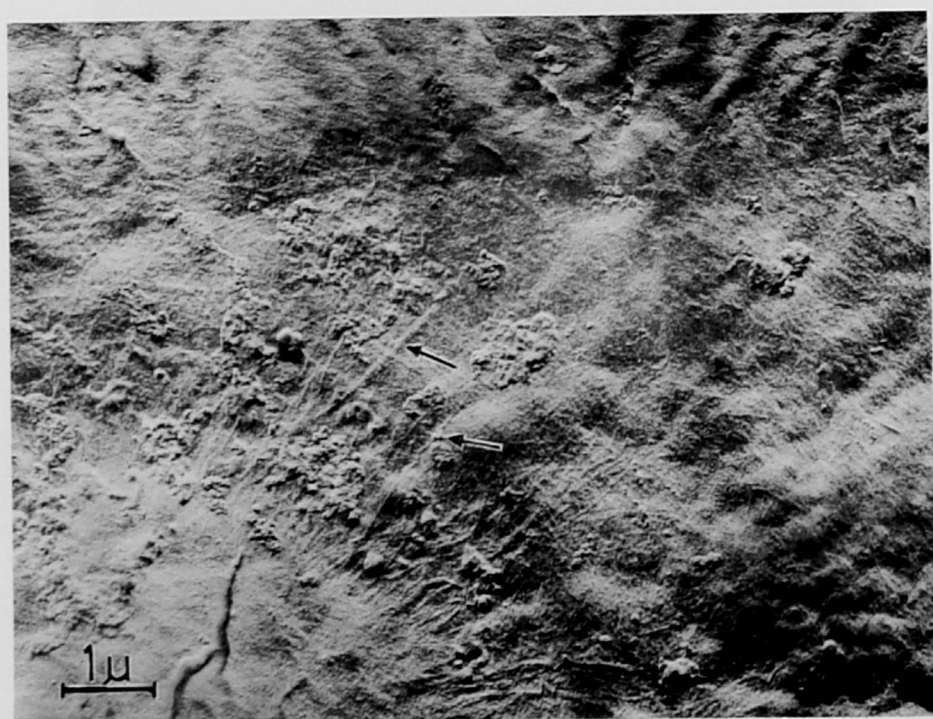


Fig. 11



Fig. 12

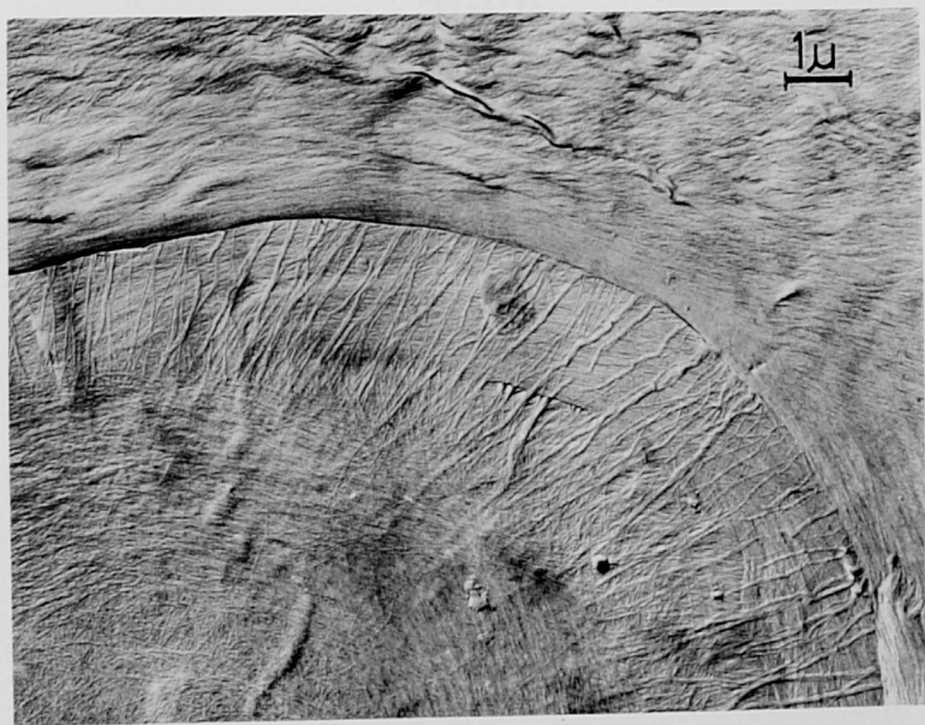


Fig. 13



Fig. 14

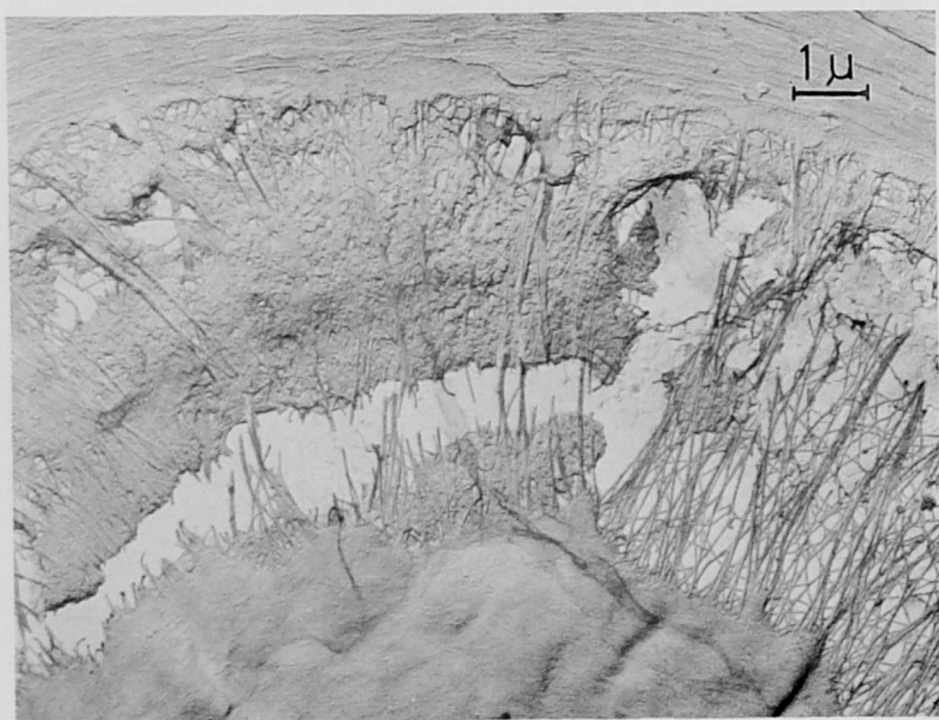


Fig. 15

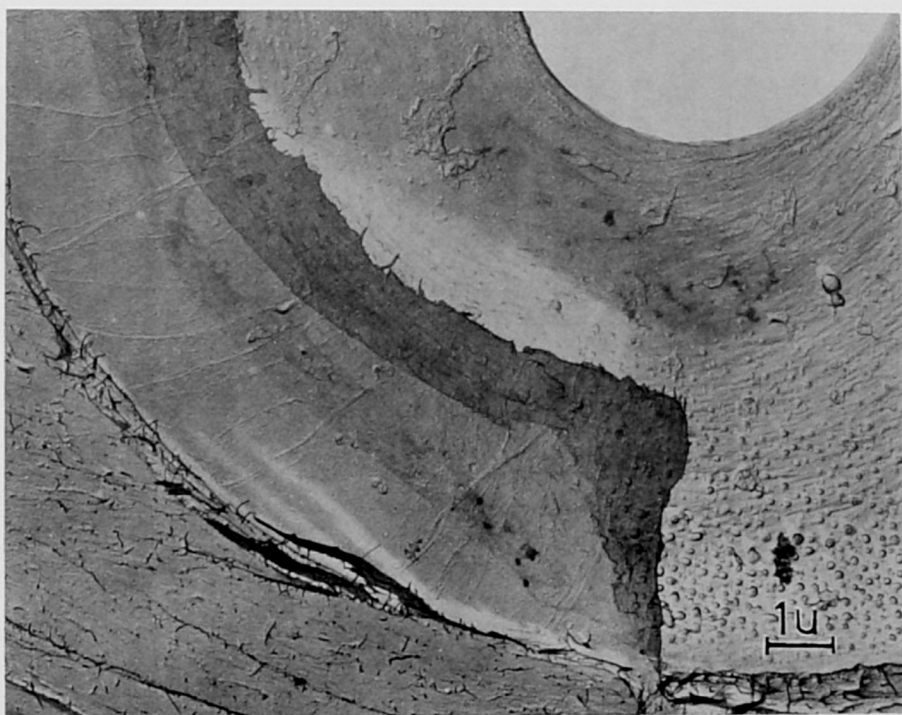


Fig. 16



Fig. 17

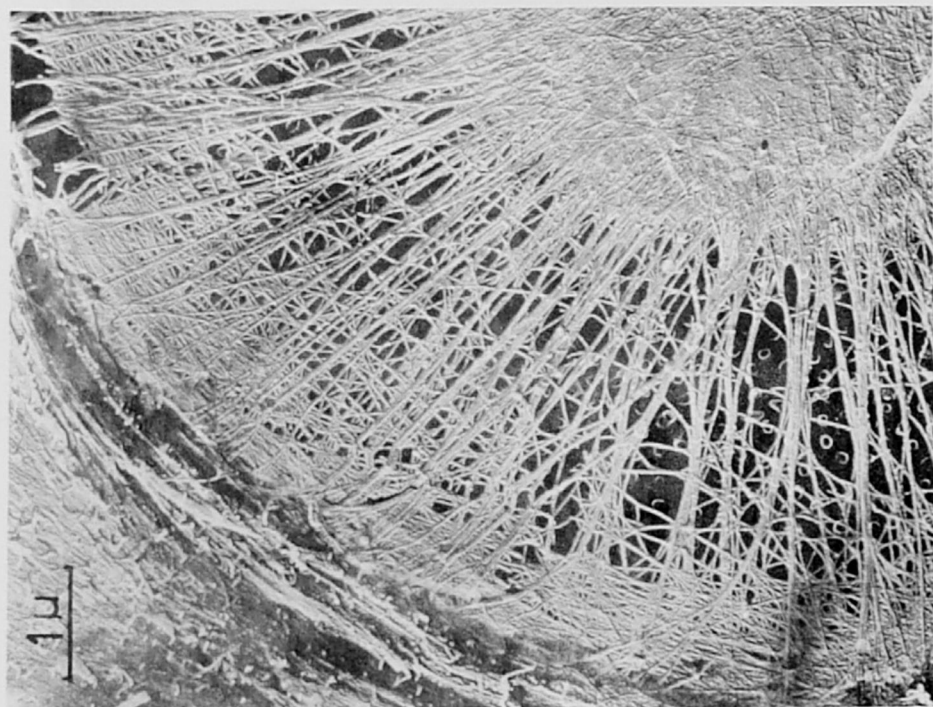


Fig. 18

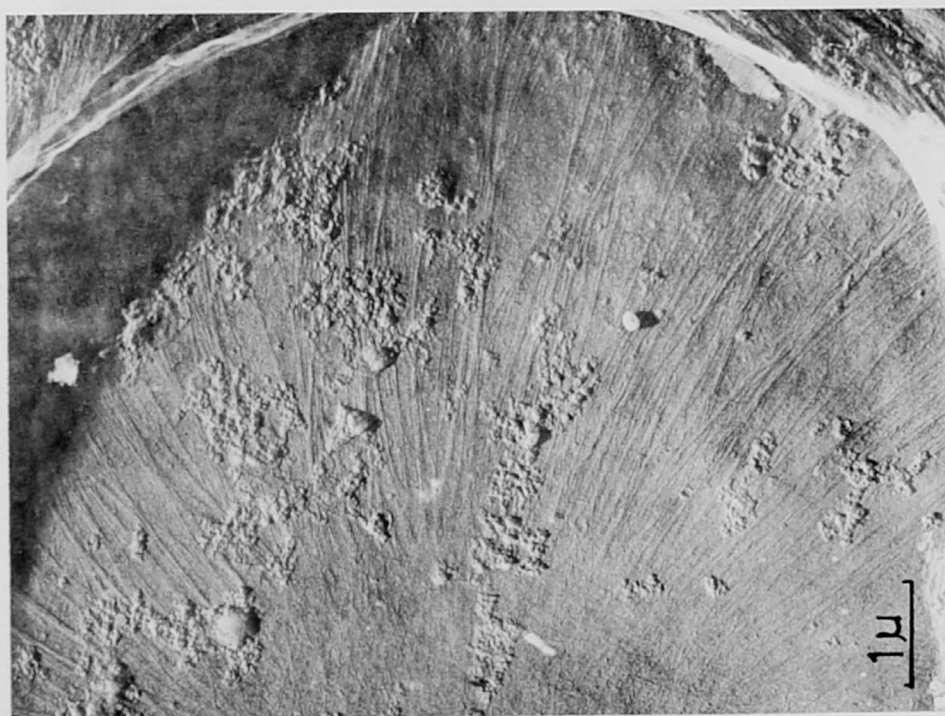


Fig. 19

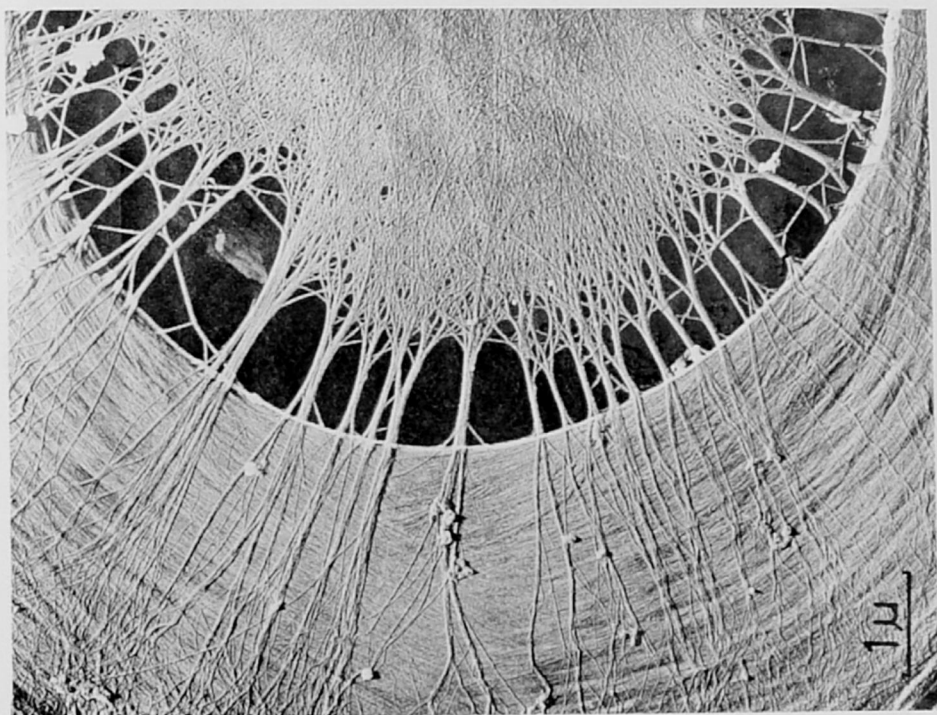


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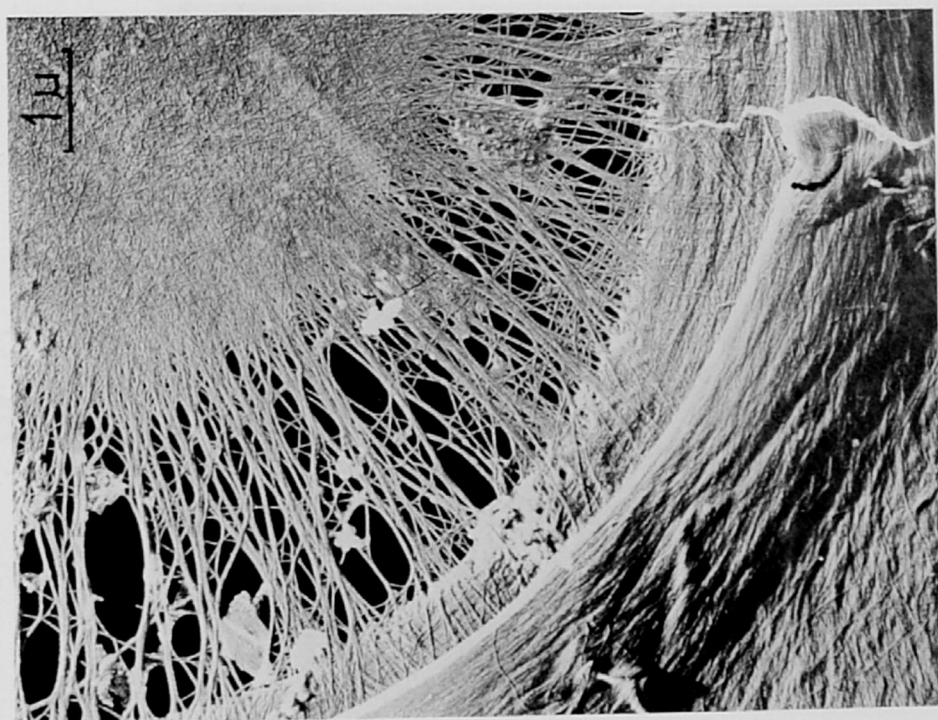


Fig. 21



Fig. 23

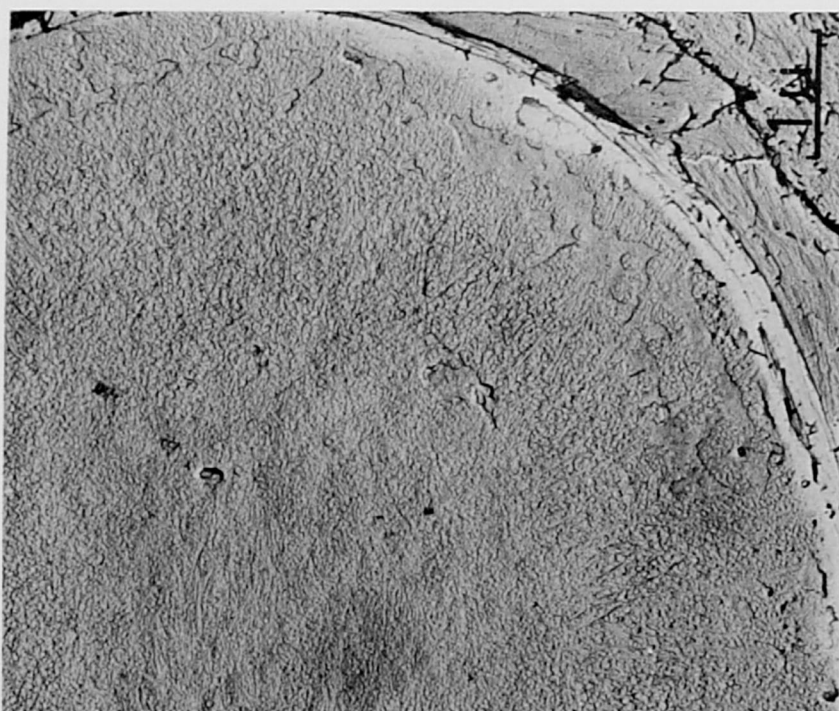


Fig. 22

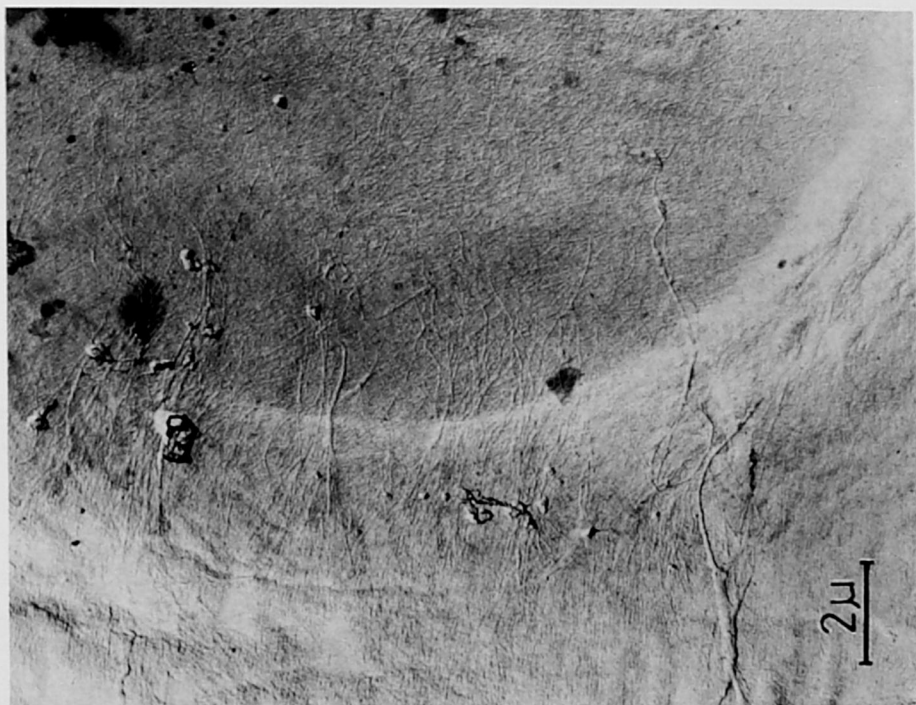


Fig. 24

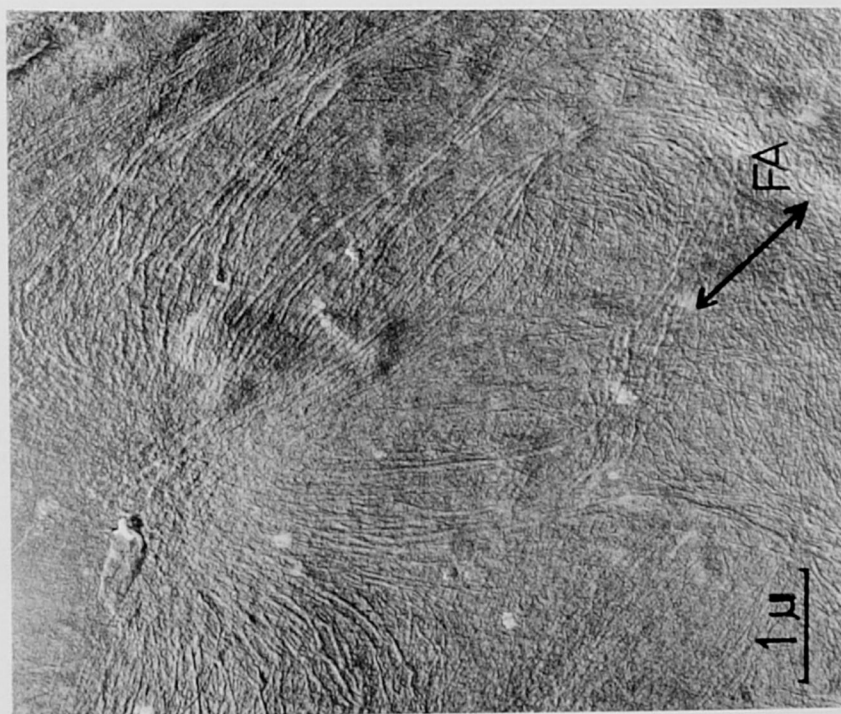


Fig. 25



Fig. 26

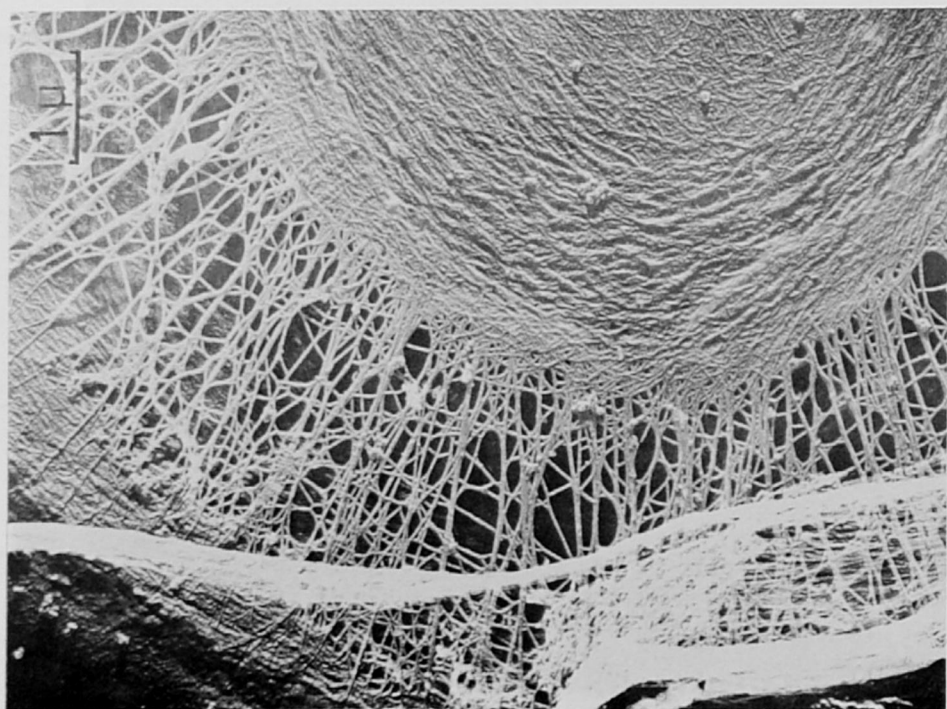


Fig. 27

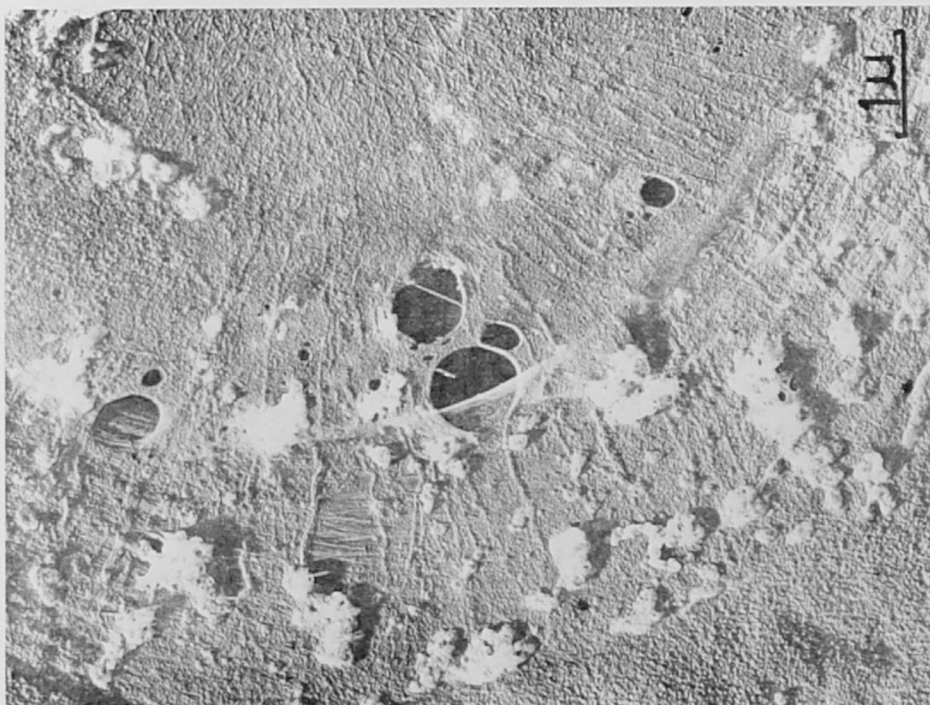


Fig. 28



Fig. 29

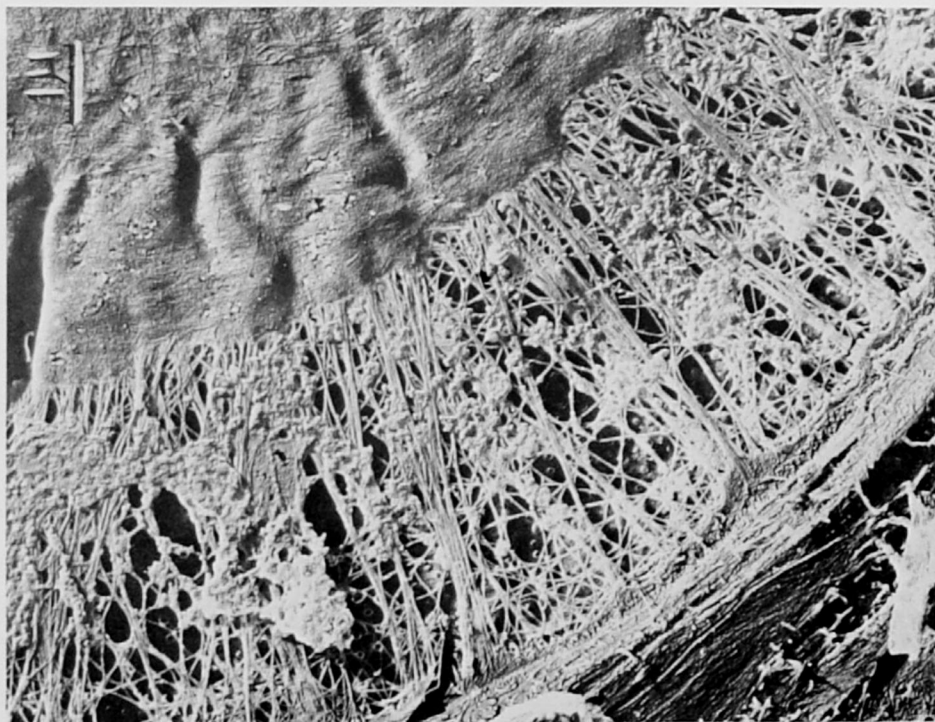


Fig. 30

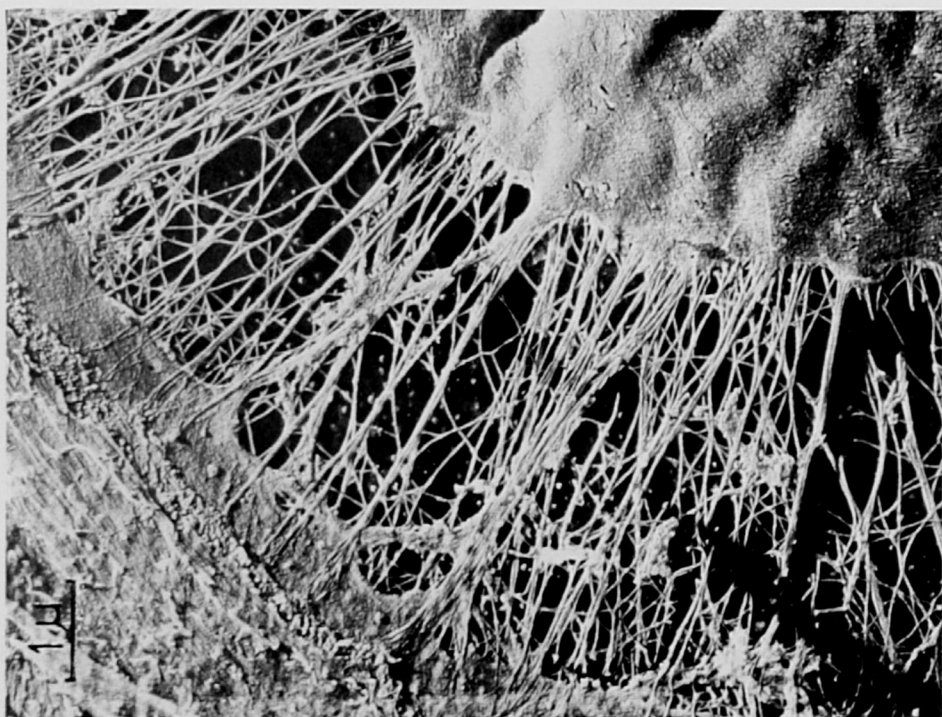


Fig. 31

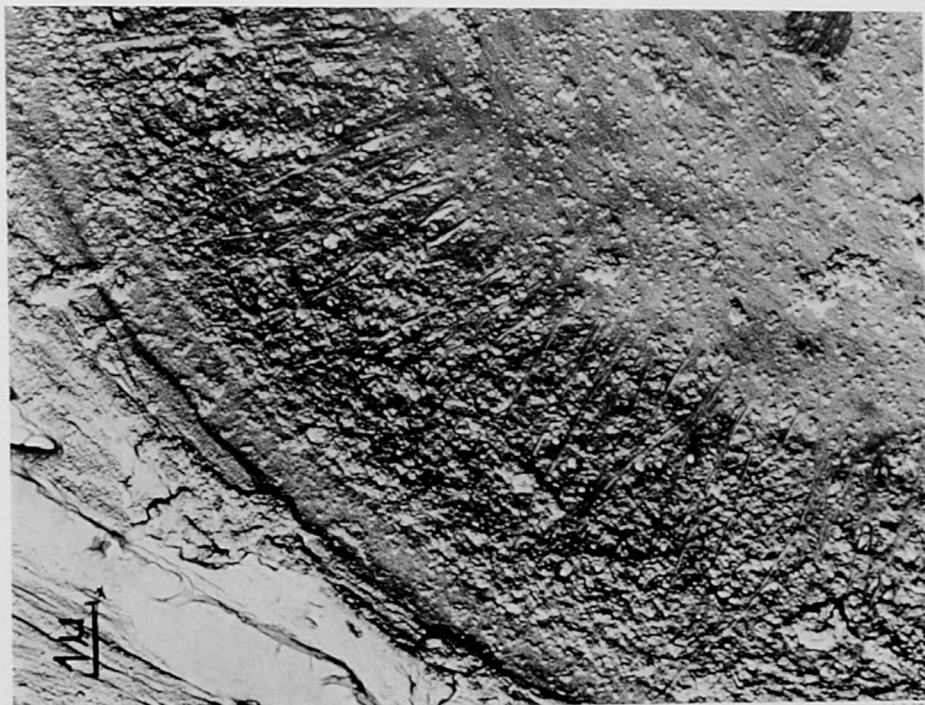


Fig. 32

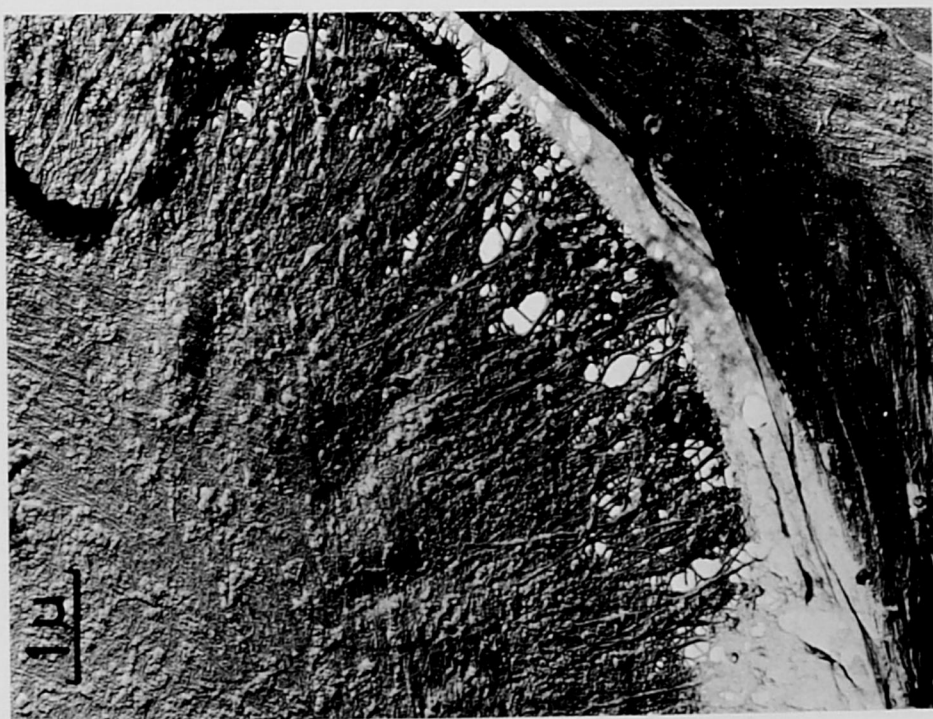


Fig. 33

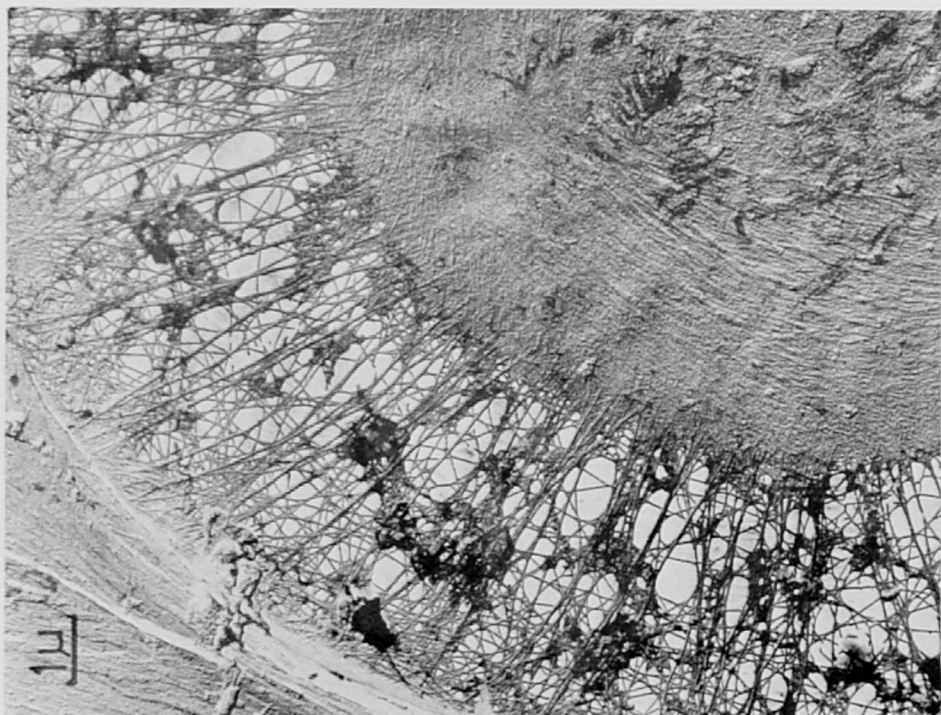


Fig. 34

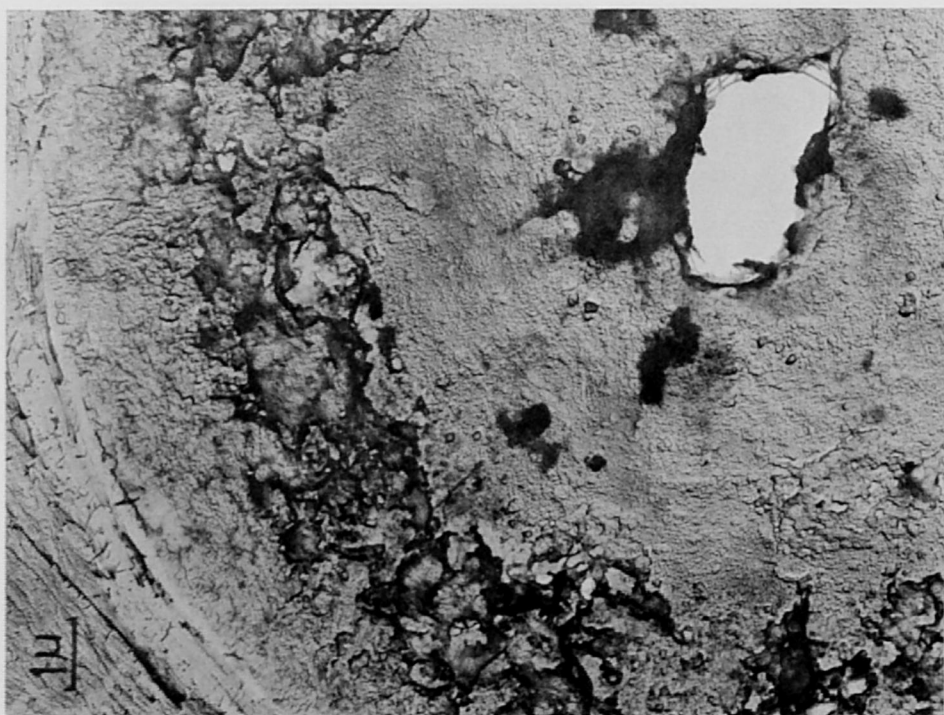


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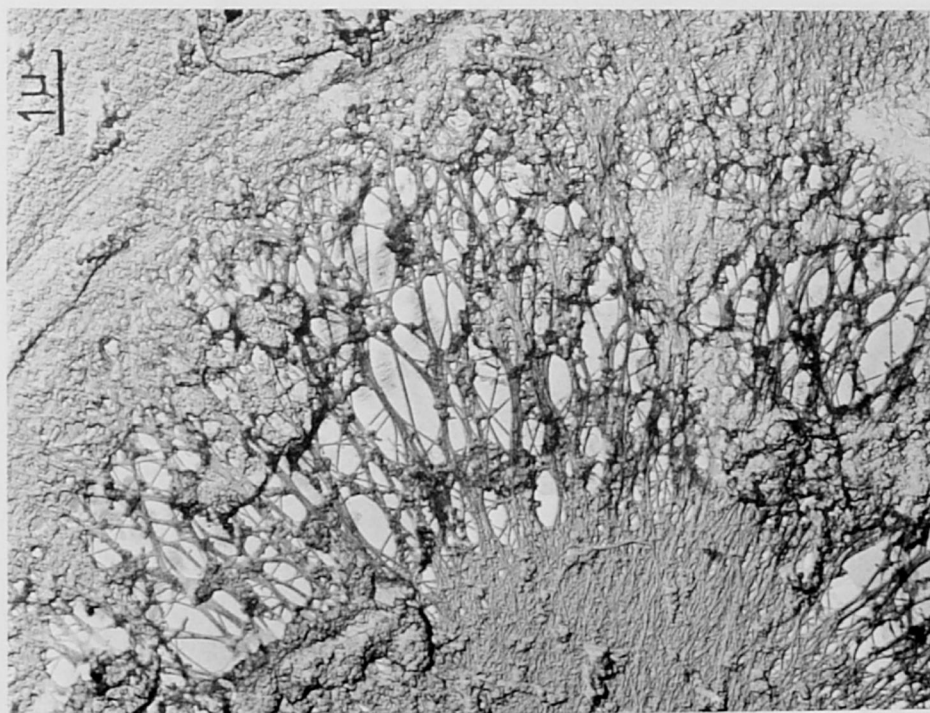


Fig. 36



Fig. 37

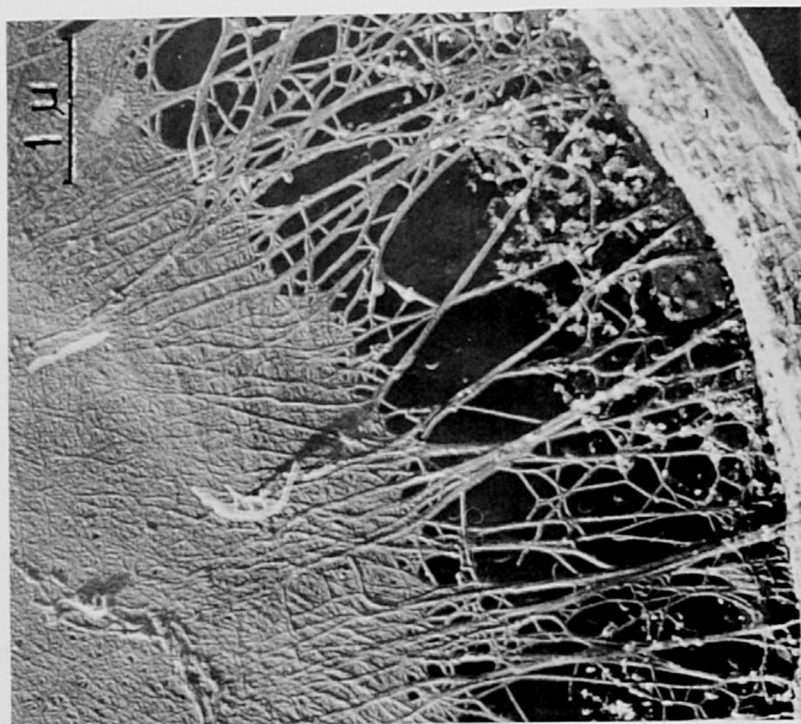


Fig. 38

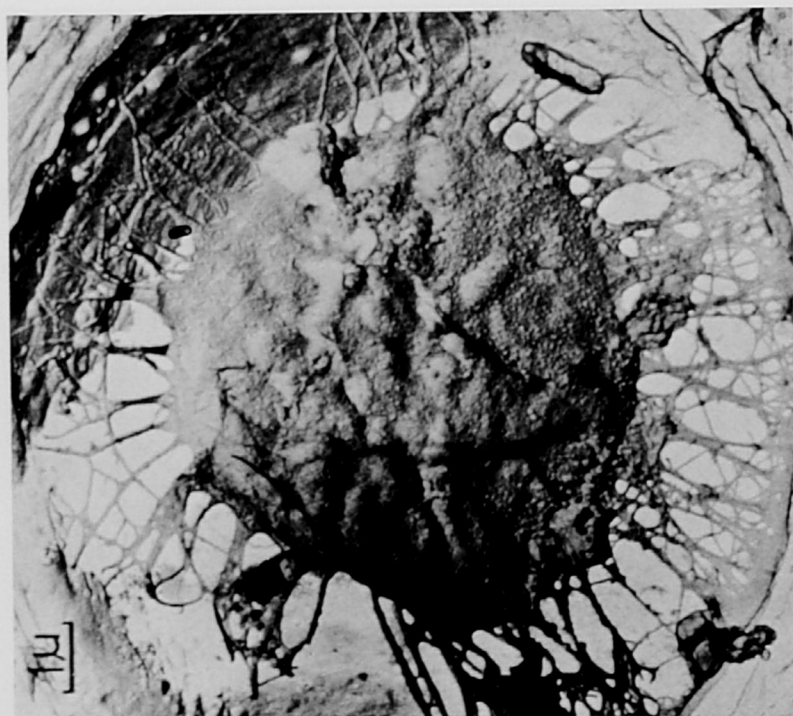


Fig. 39

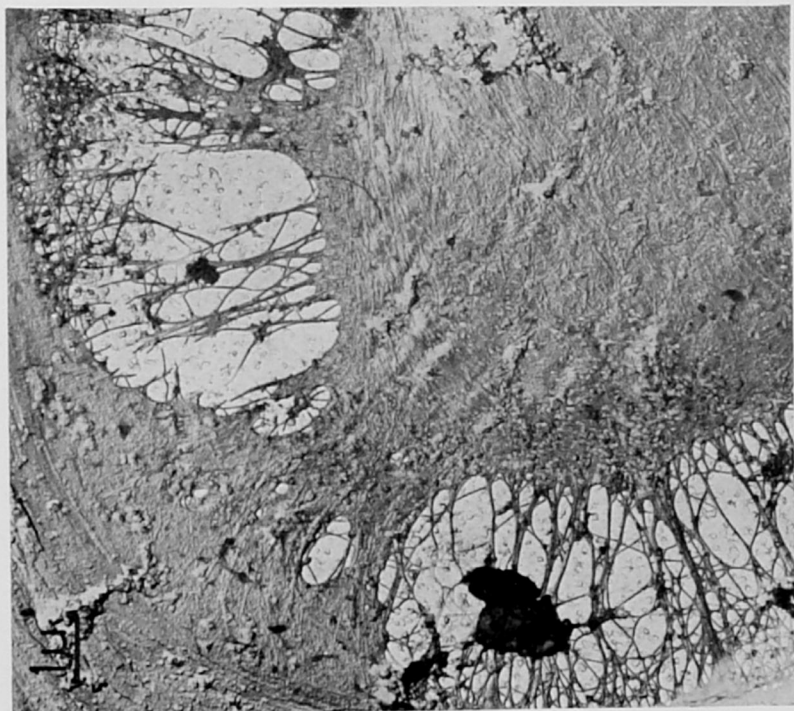


Fig. 40



Fig. 41



Fig. 42

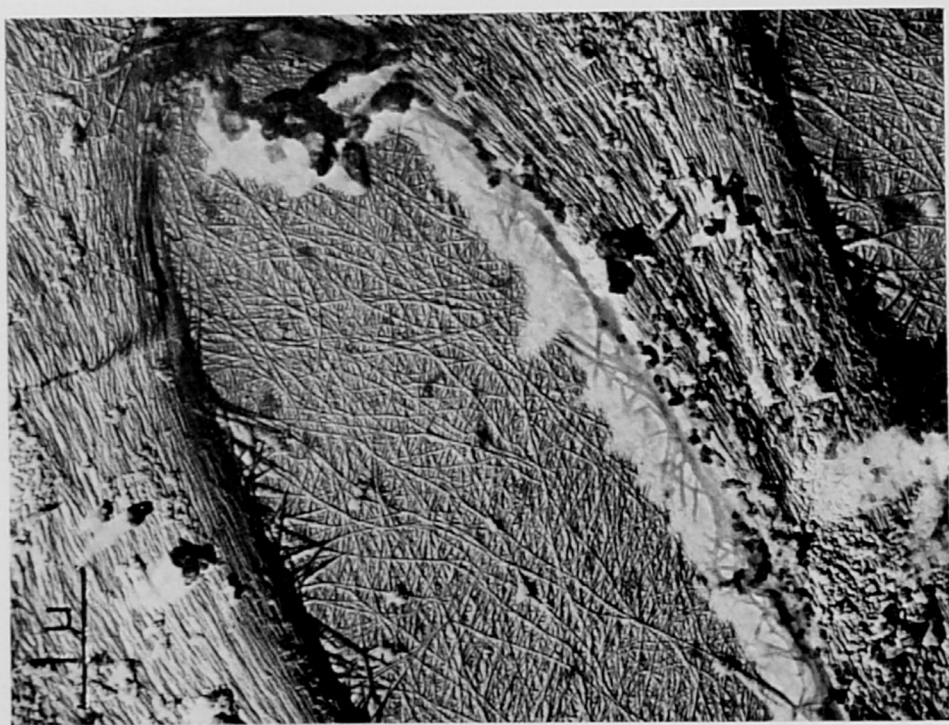


Fig. 43



Fig. 44

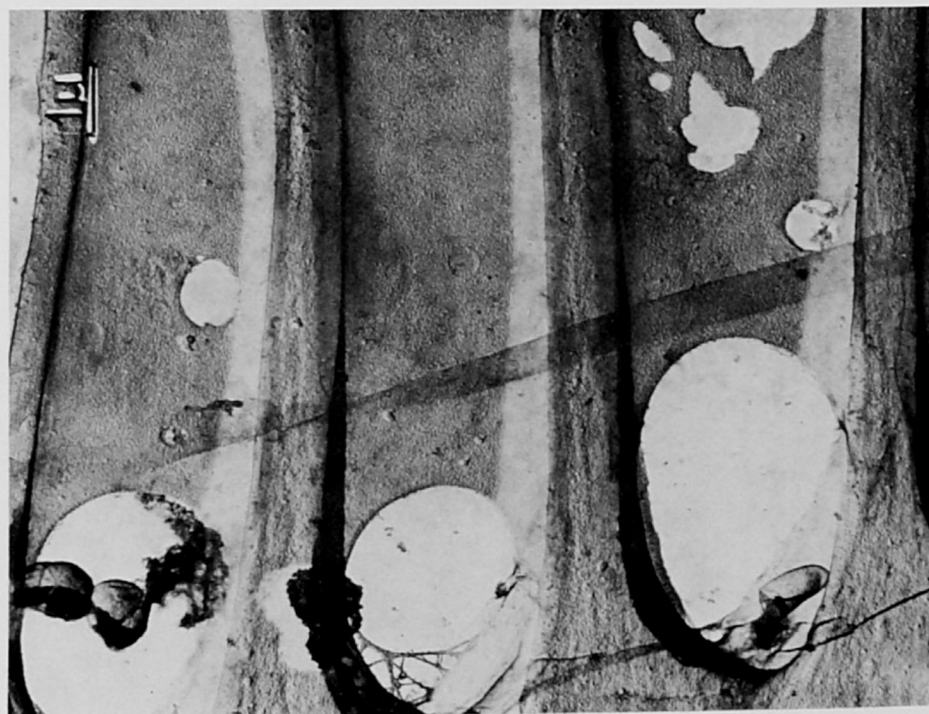


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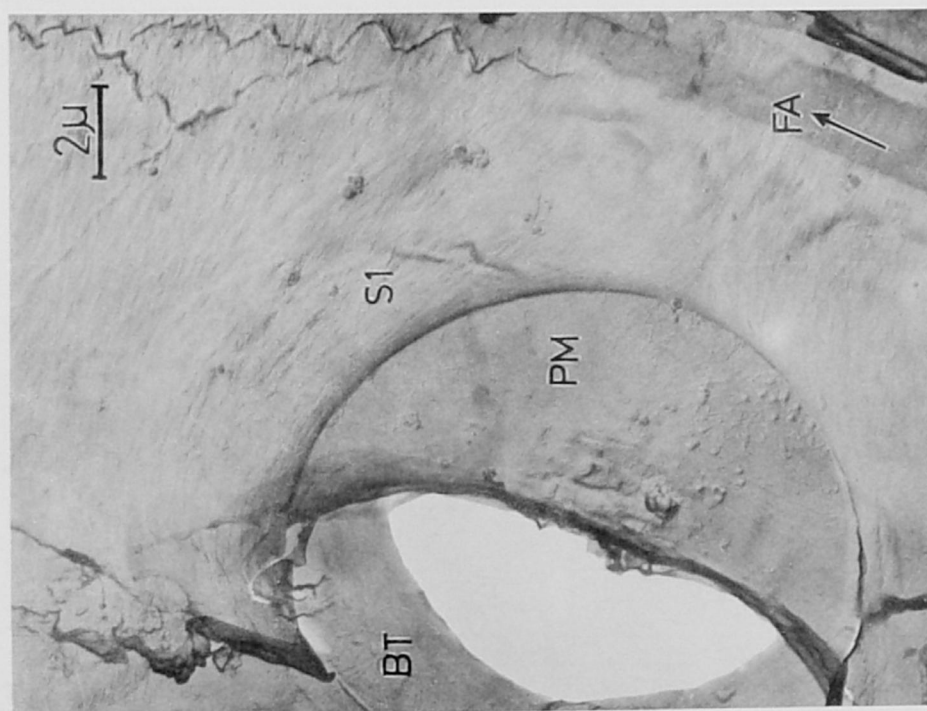


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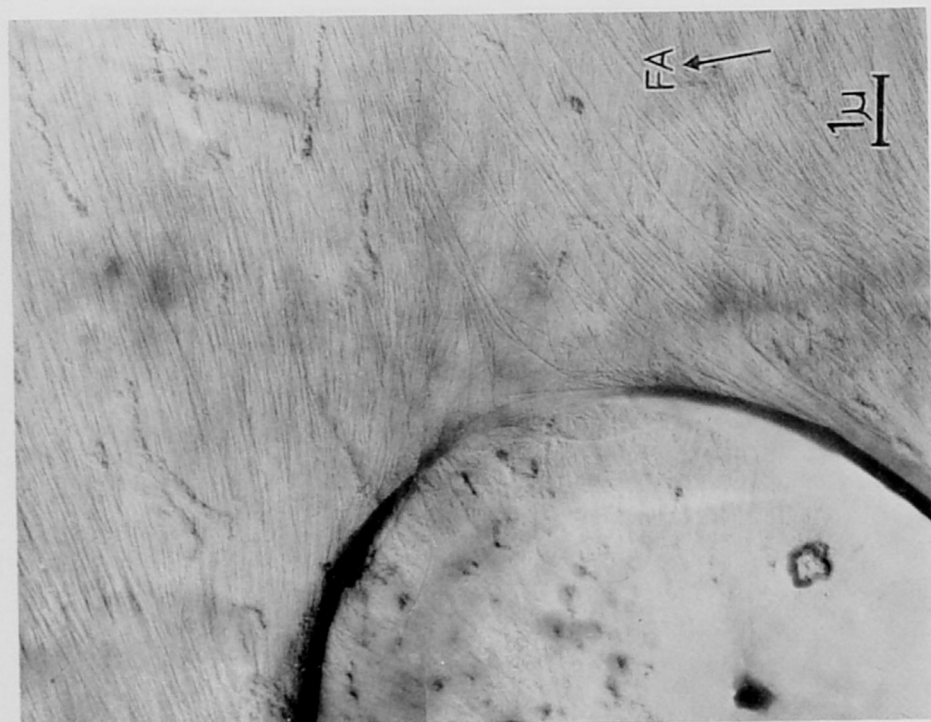


Fig. 47

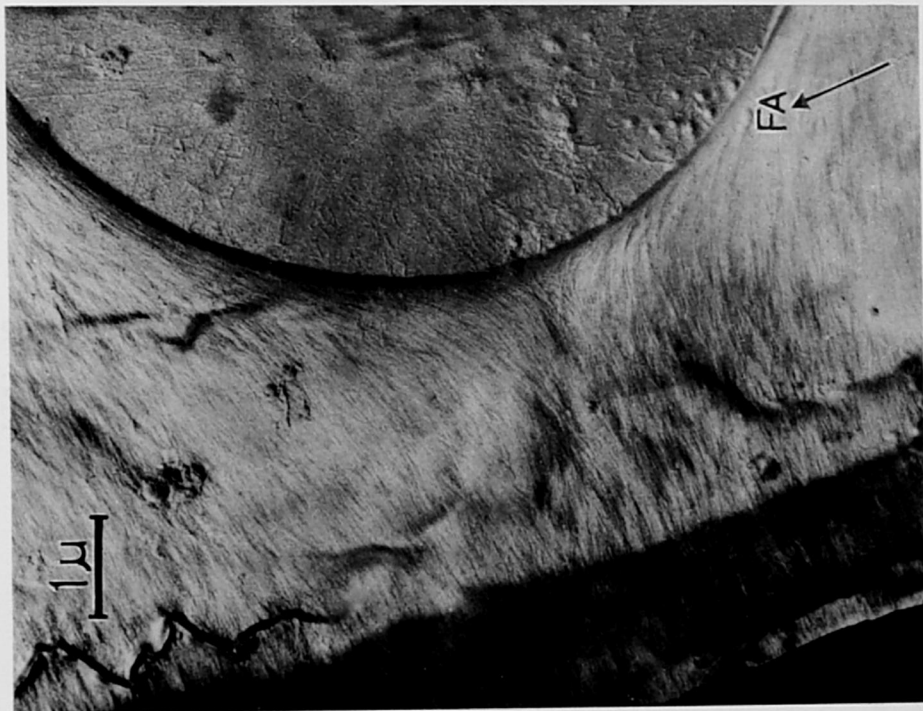


Fig. 48

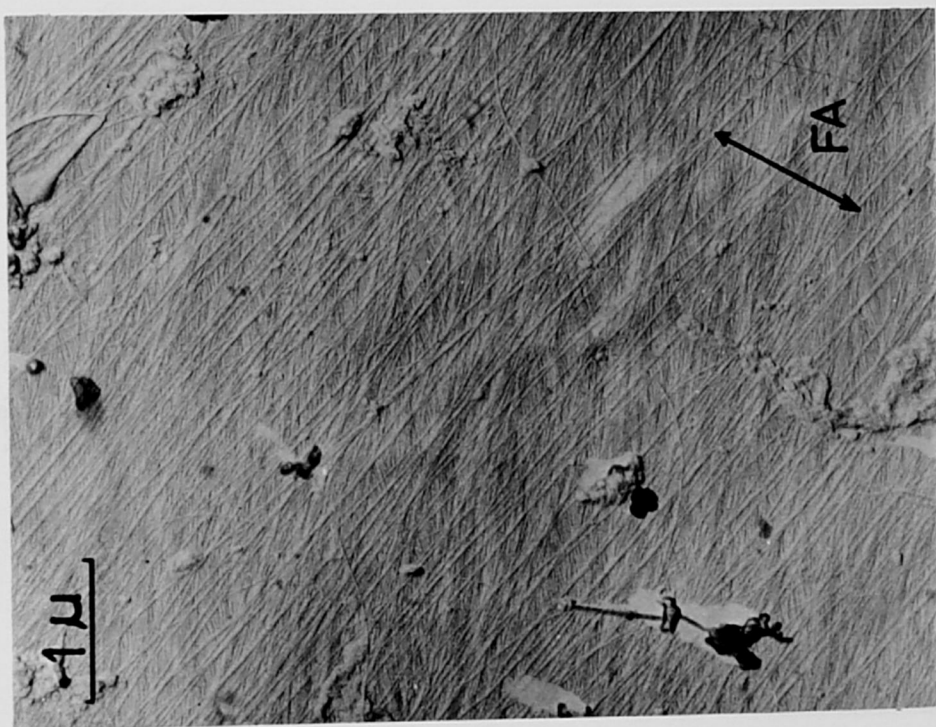


Fig. 49



Fig. 50

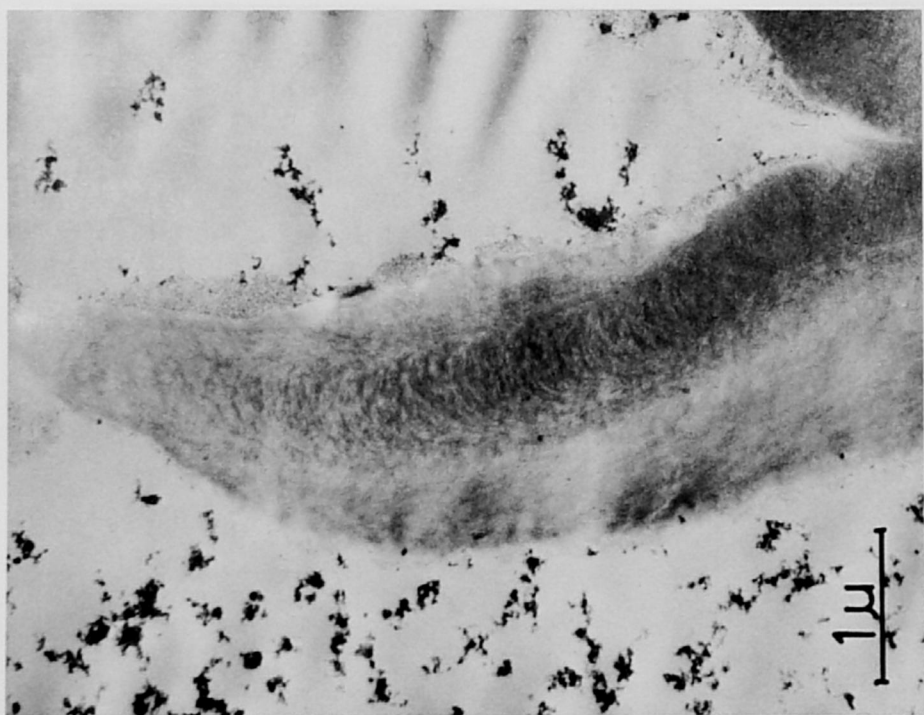


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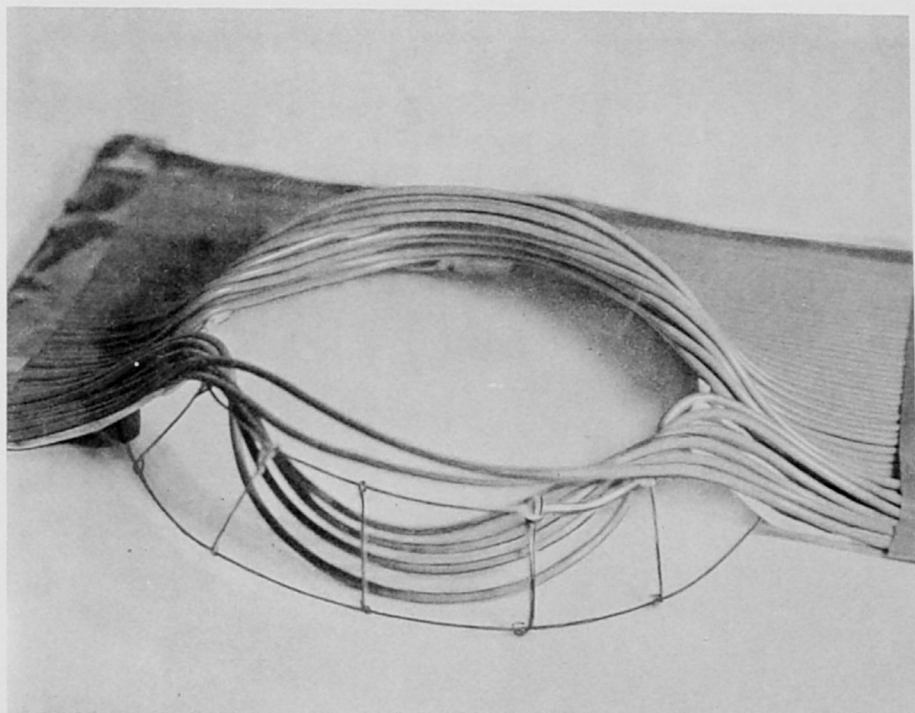


Fig. 52

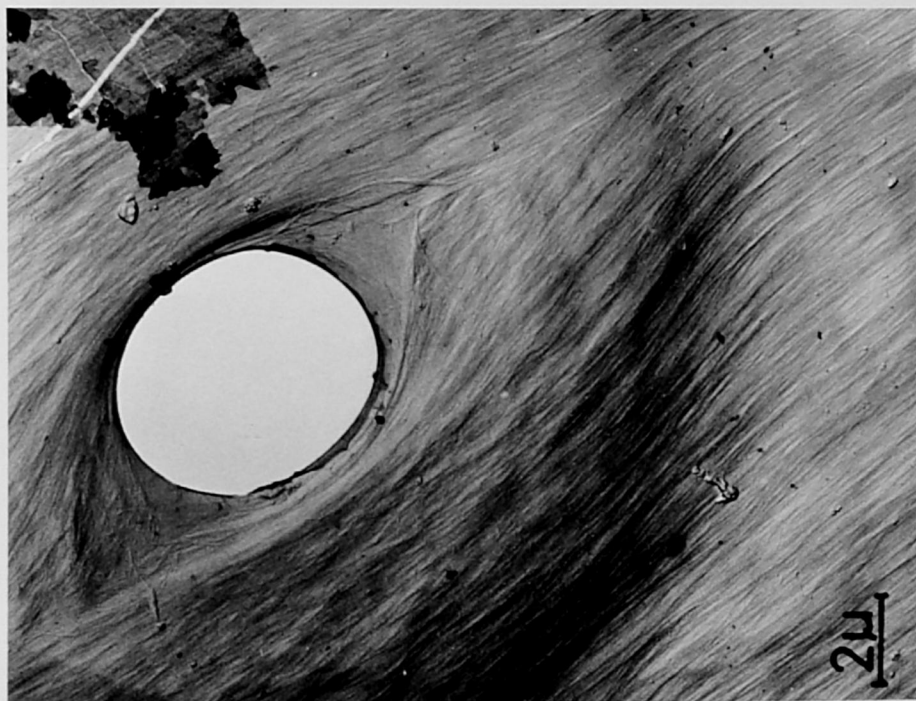


Fig. 53



Fig. 54



Fig. 55

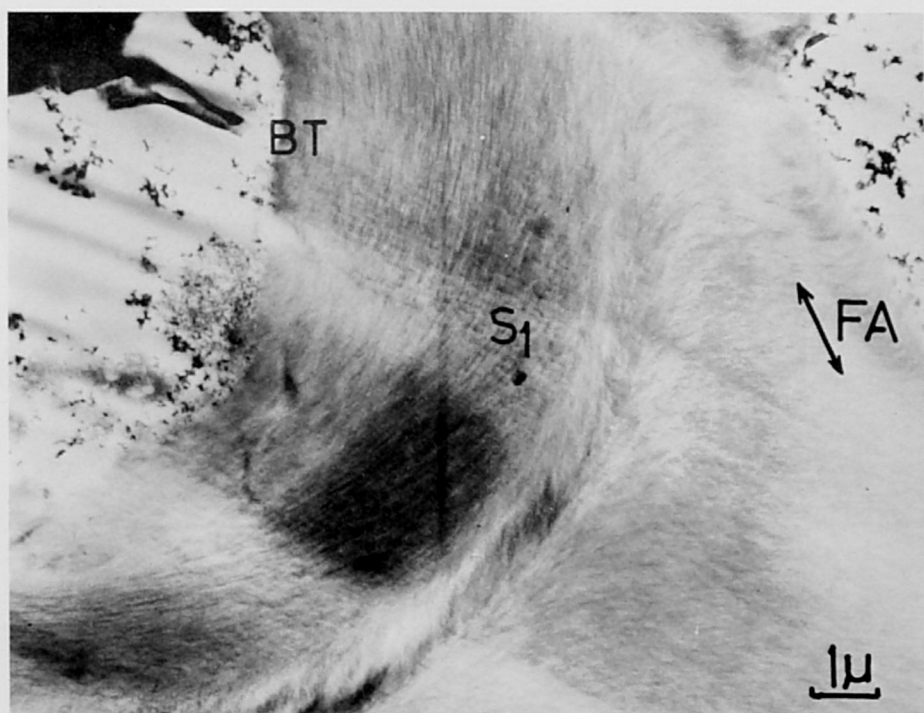


Fig. 56

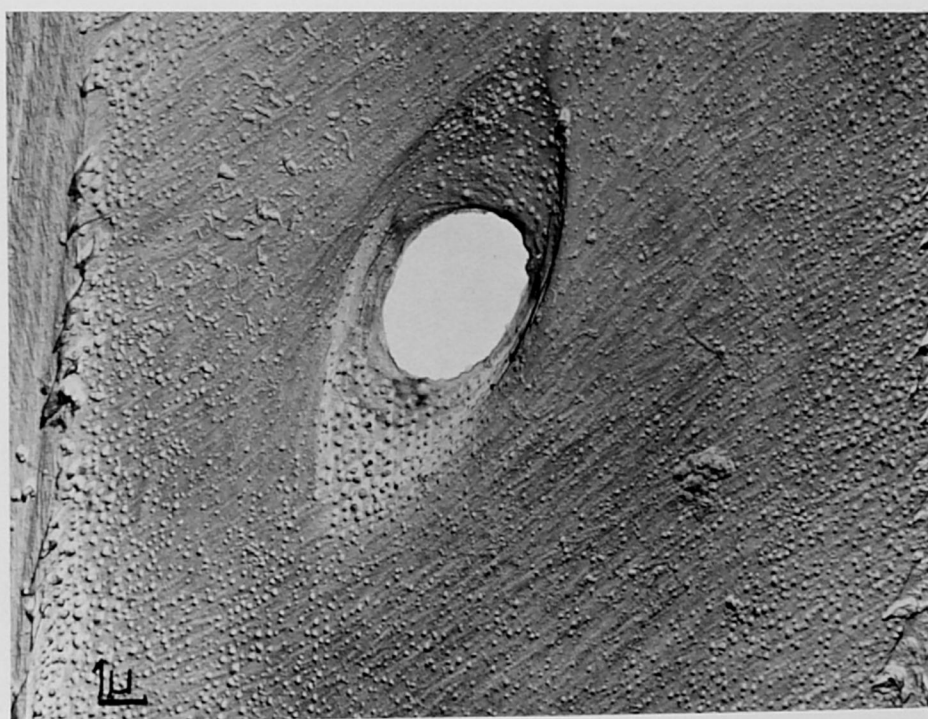


Fig. 57

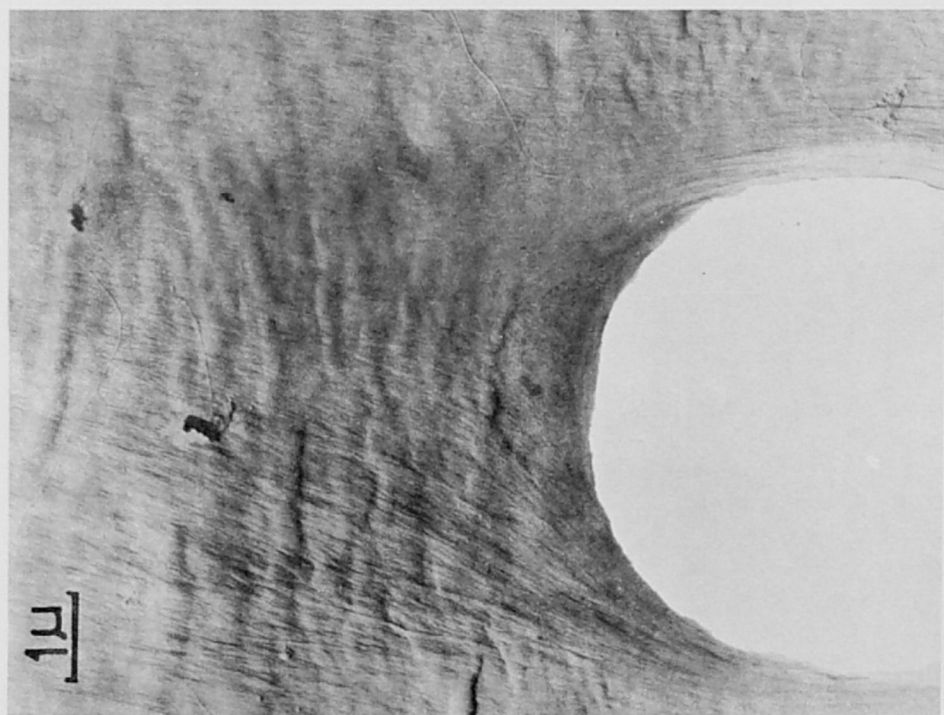


Fig. 58



Fig. 59



Fig. 60

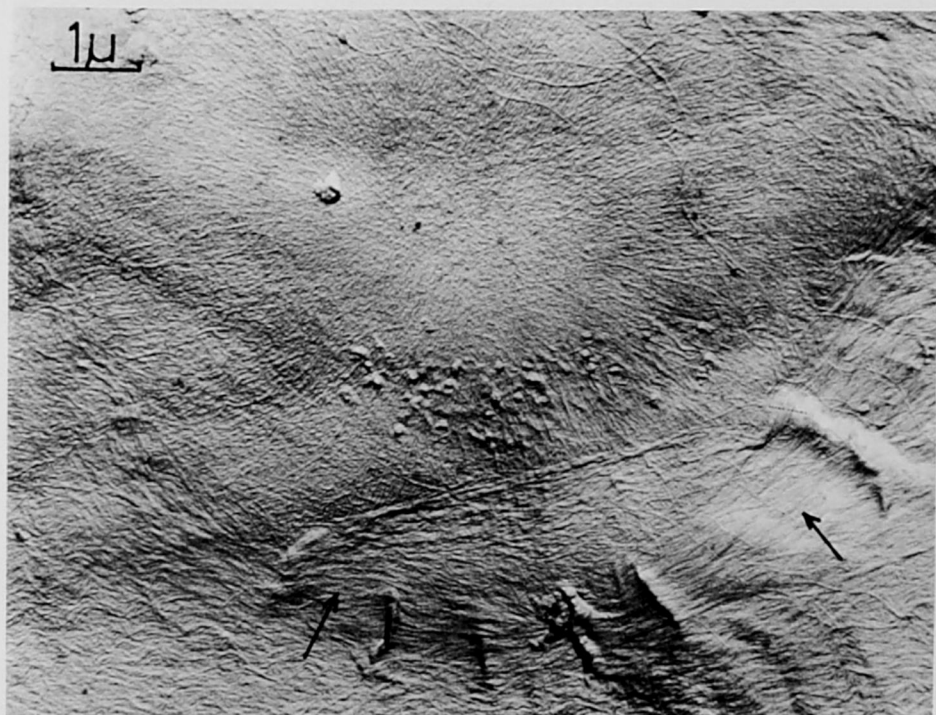


Fig. 61



Fig. 62



Fig. 63

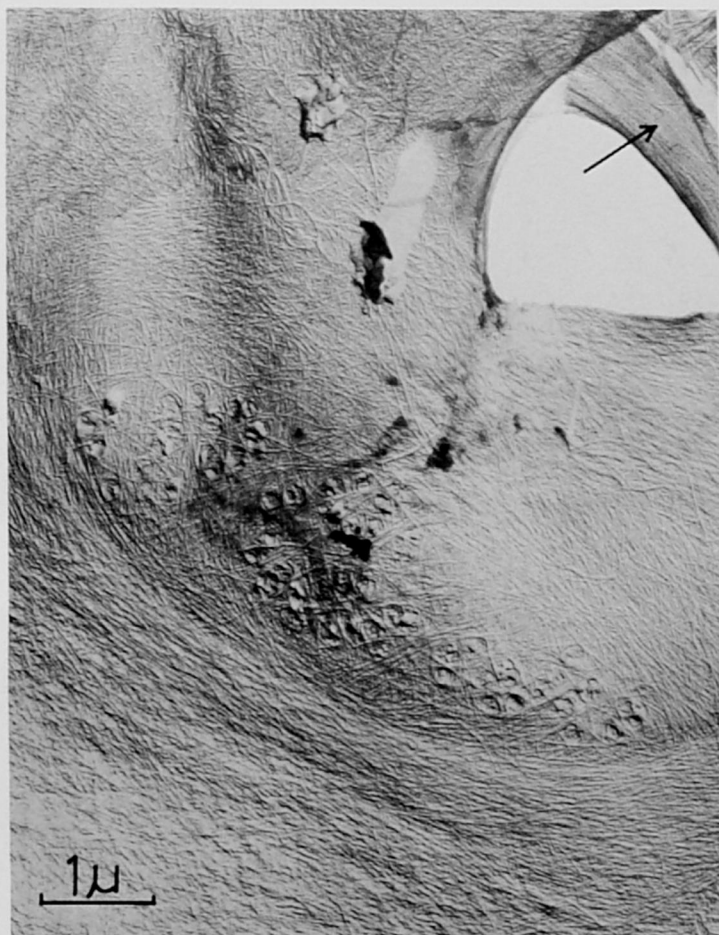


Fig. 64



Fig. 65

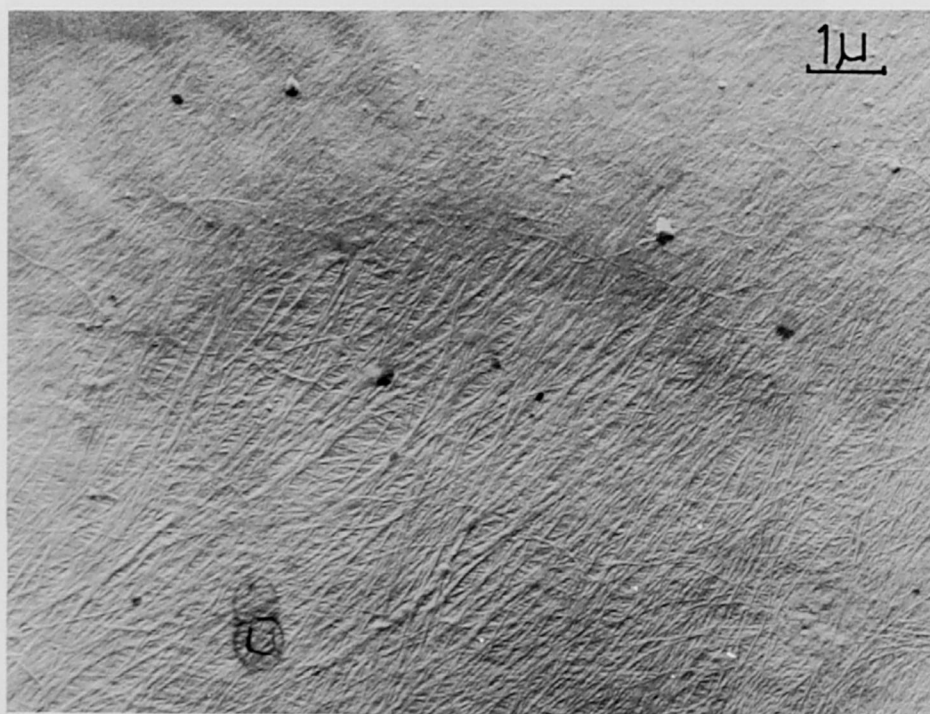


Fig. 66



Fig. 67

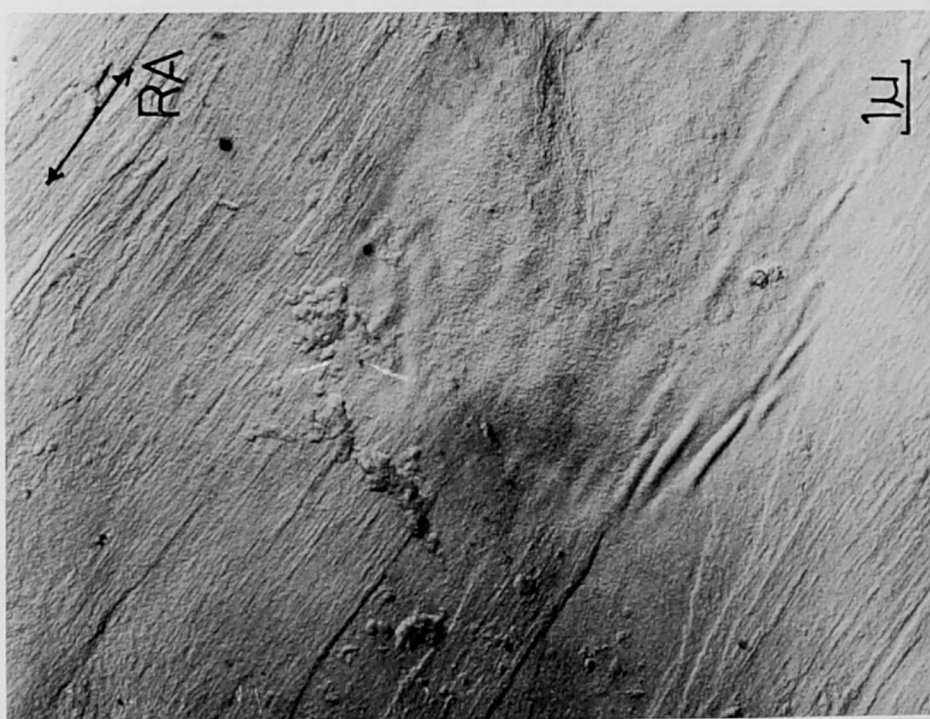


Fig. 68

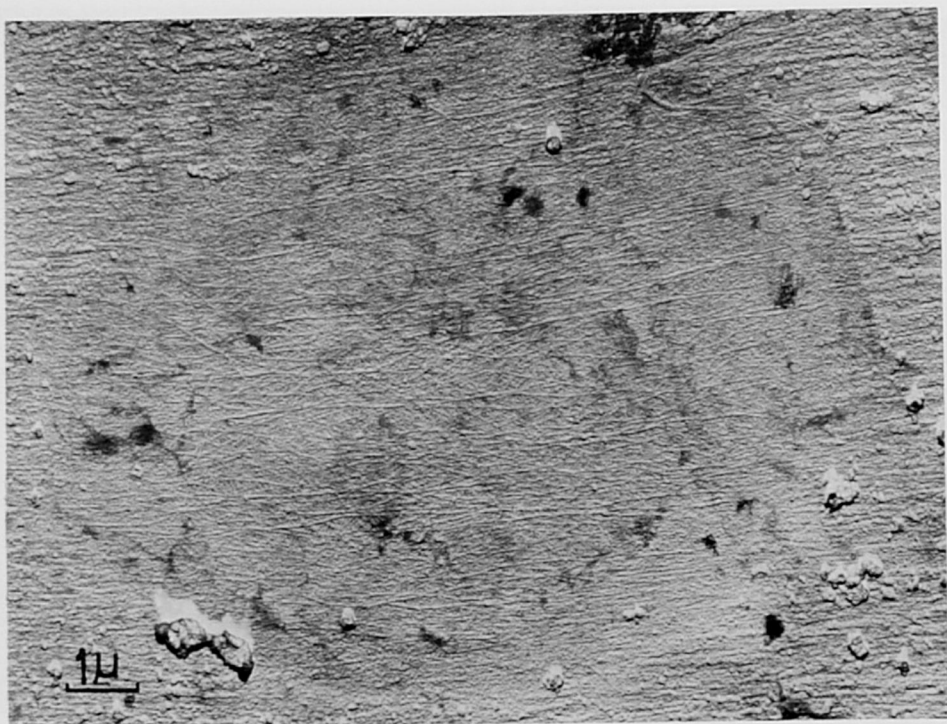


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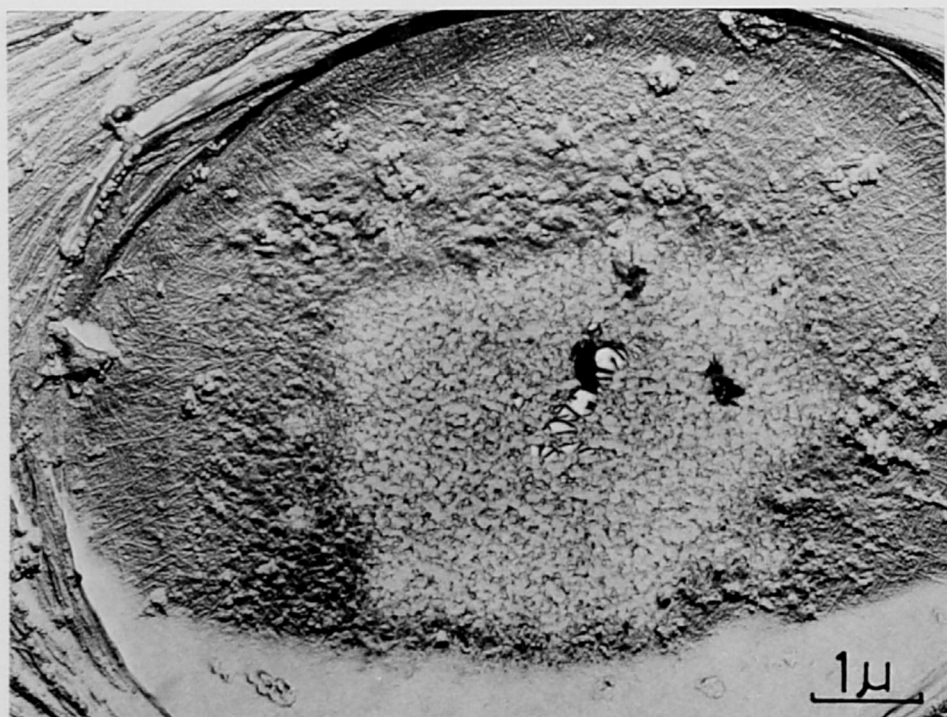


Fig. 70

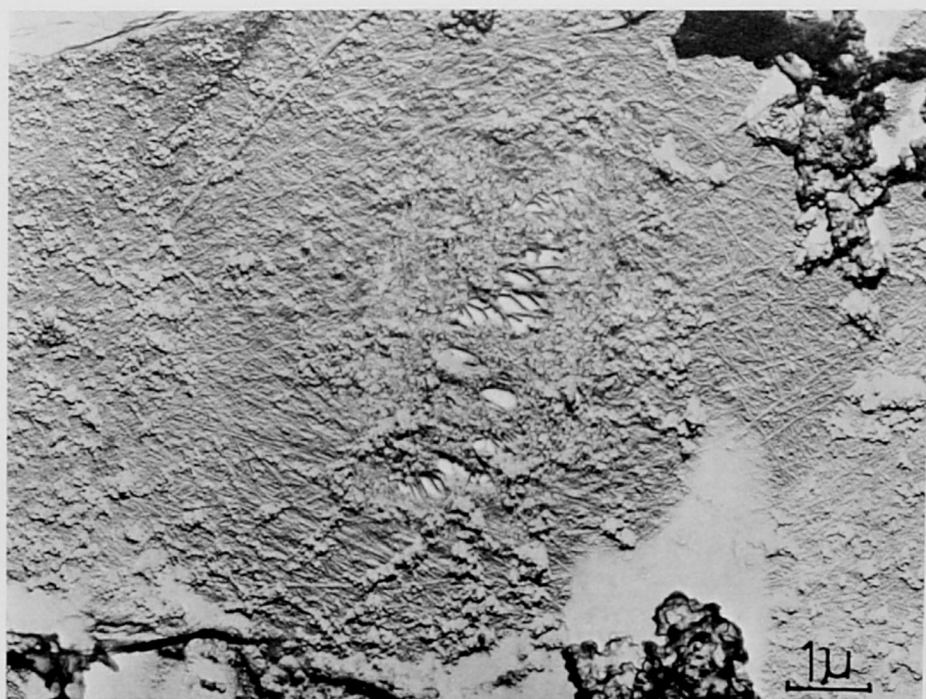


Fig. 71

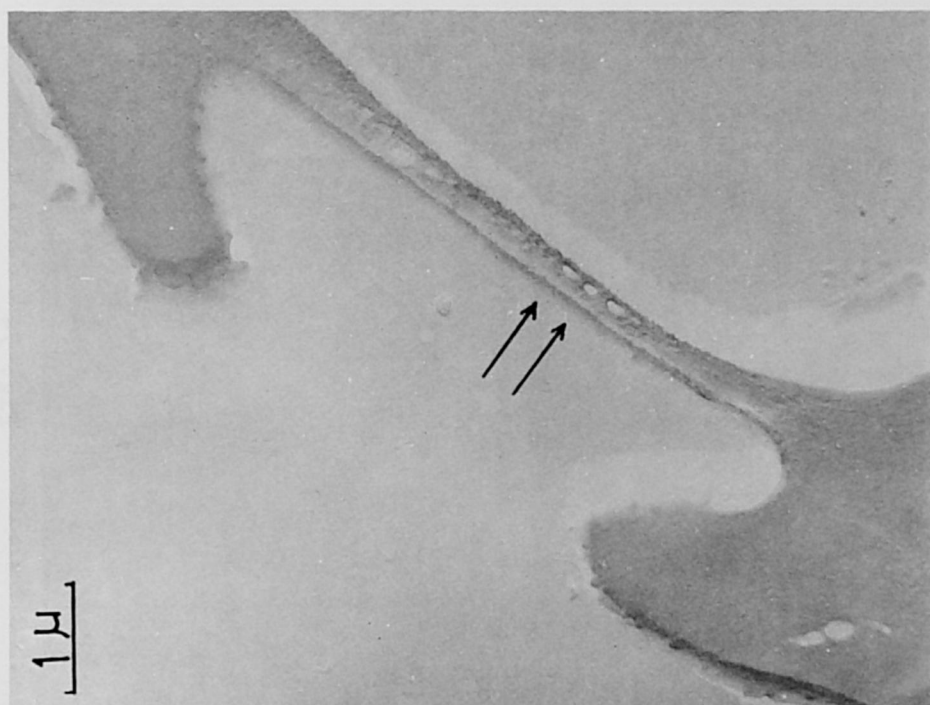


Fig. 72

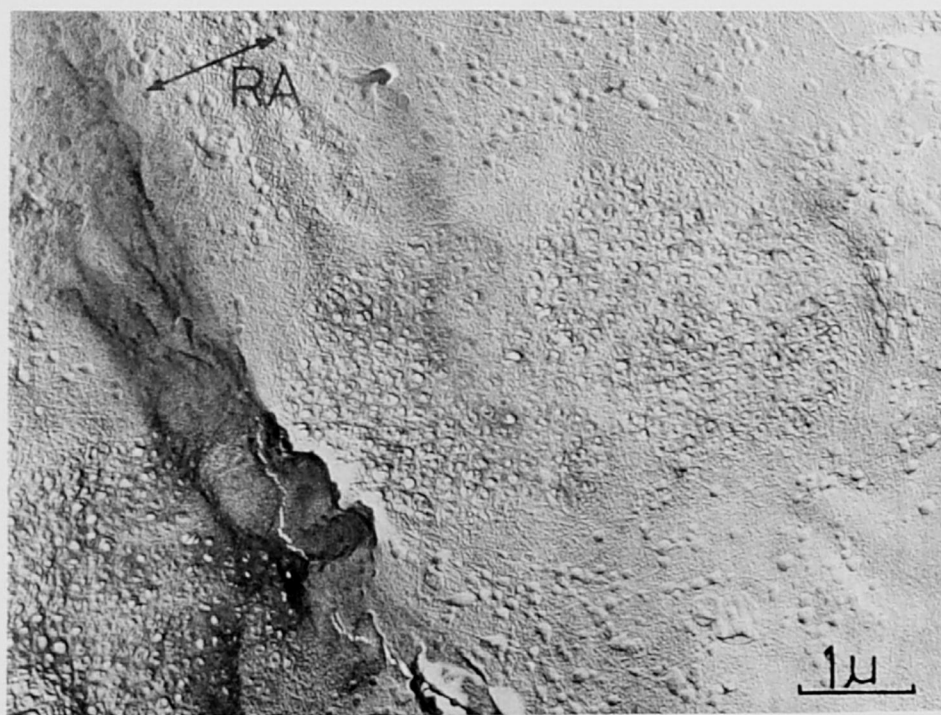


Fig. 73

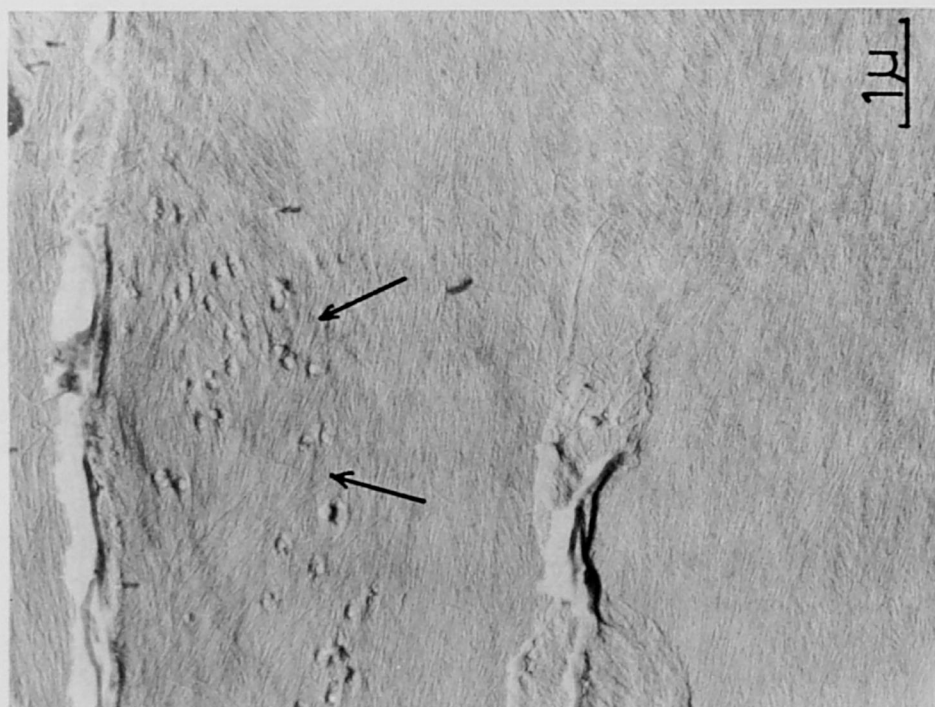


Fig. 74

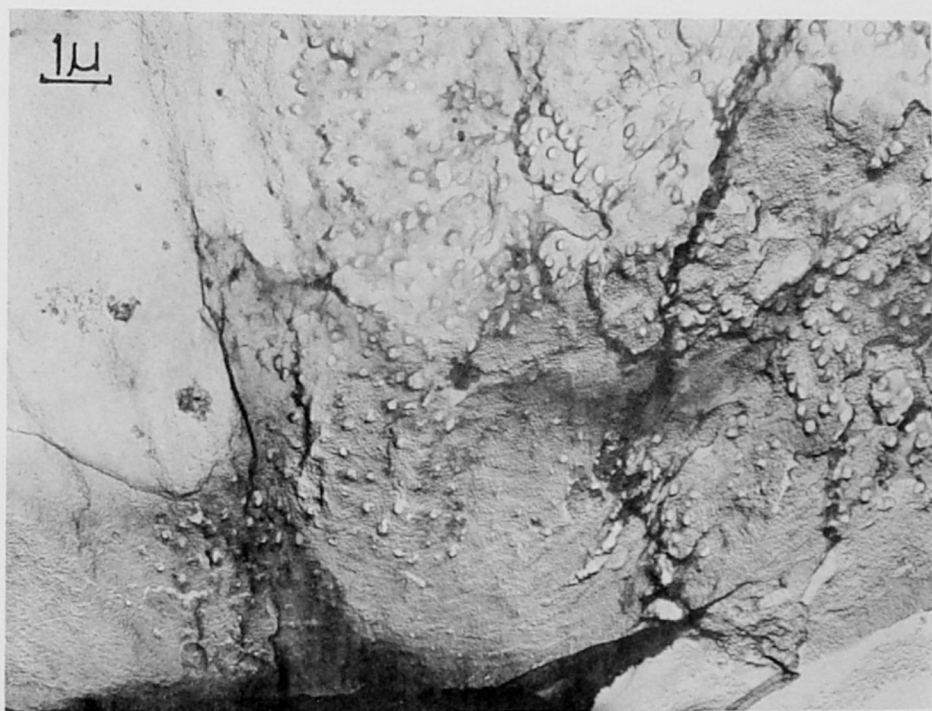


Fig. 75

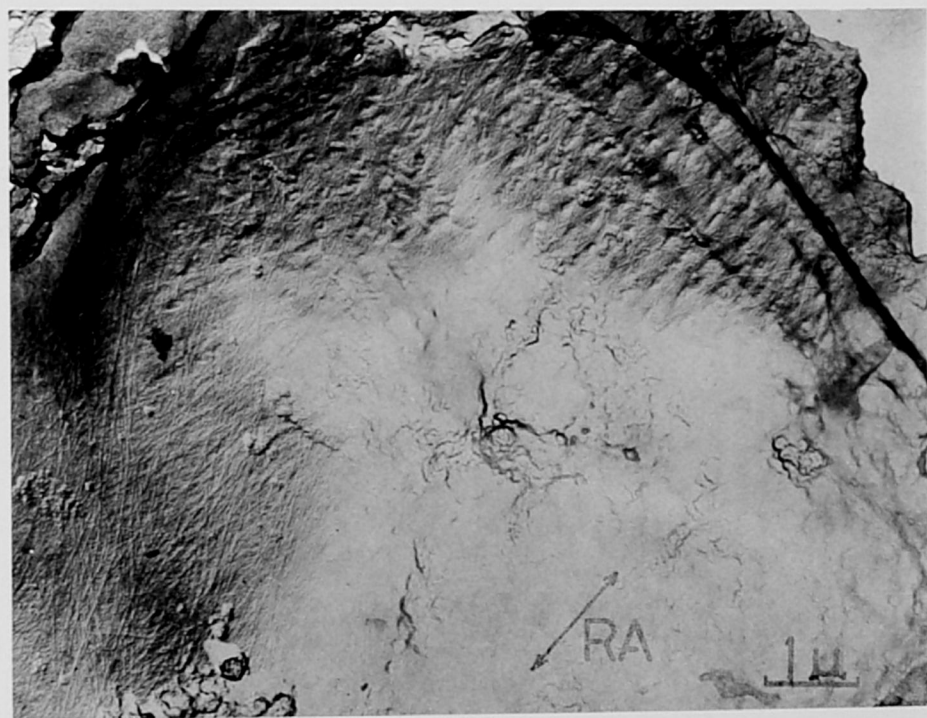


Fig. 76

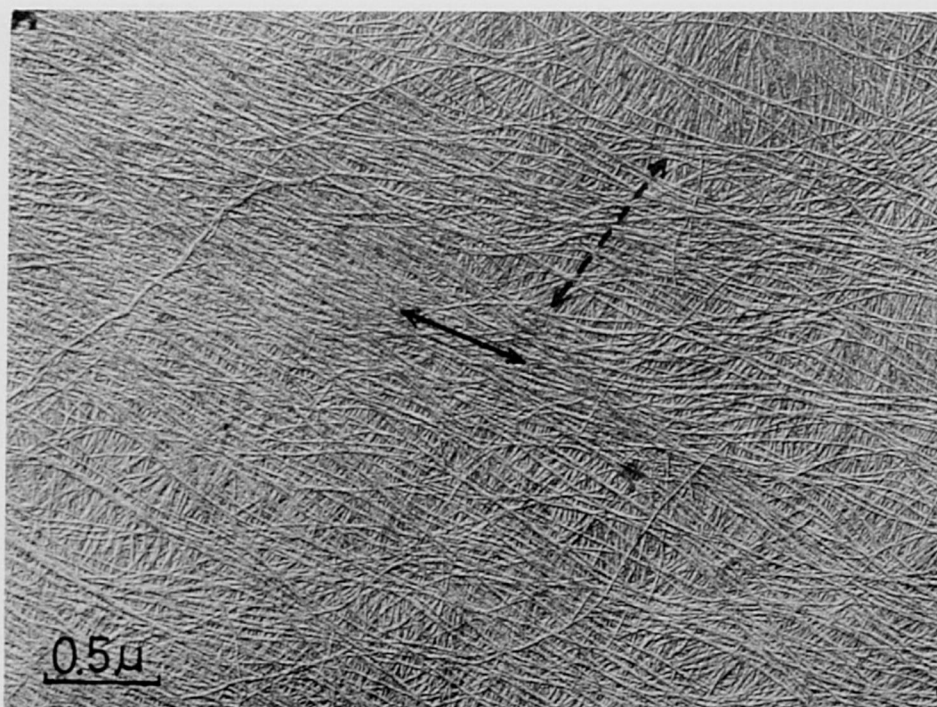


Fig. 77

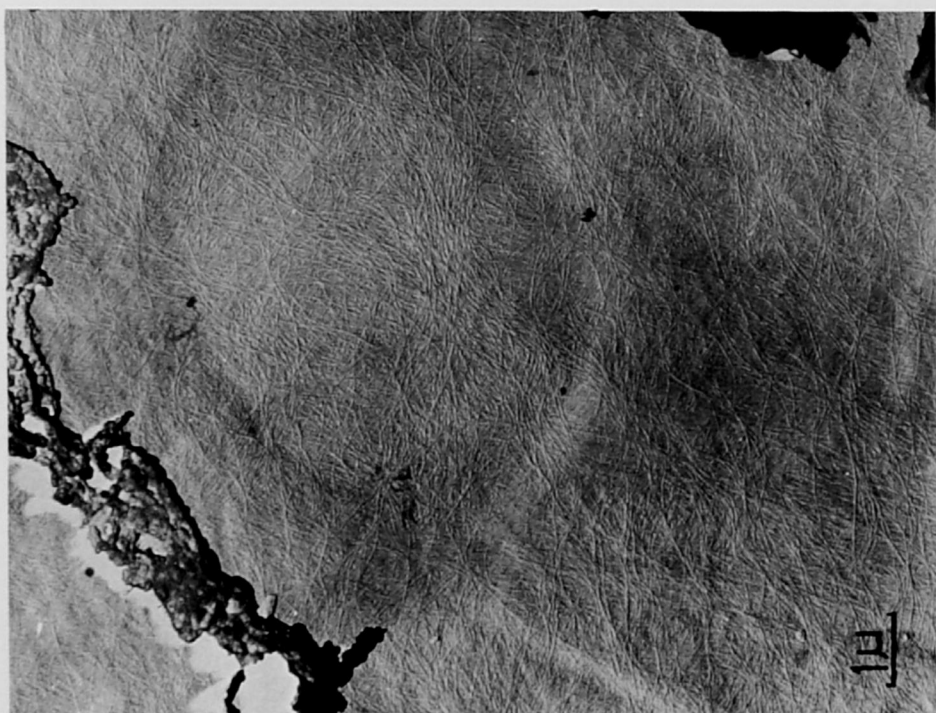


Fig. 78

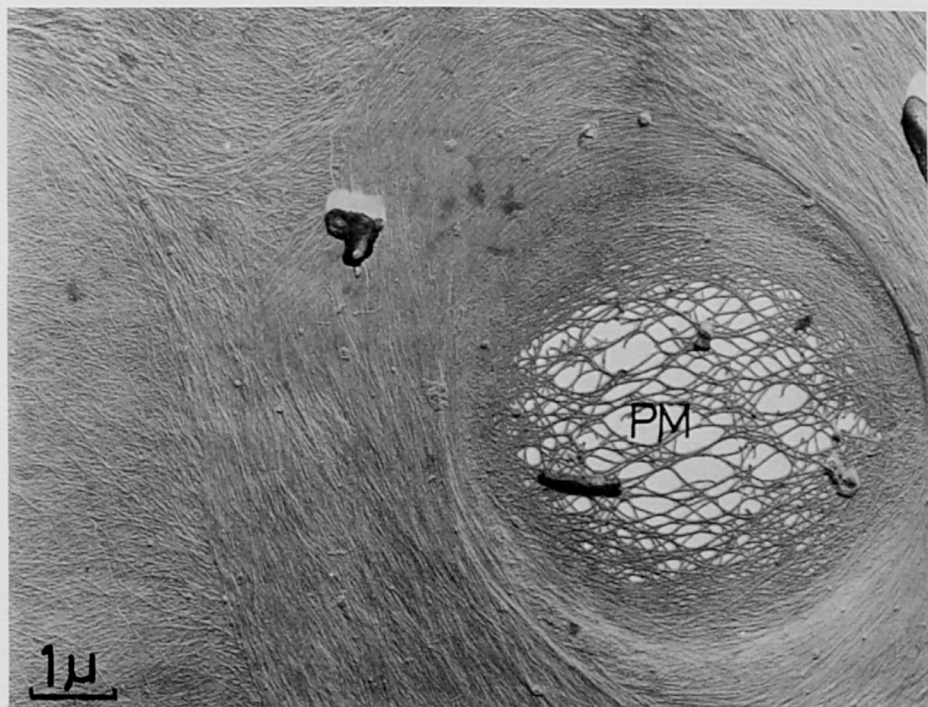


Fig. 79

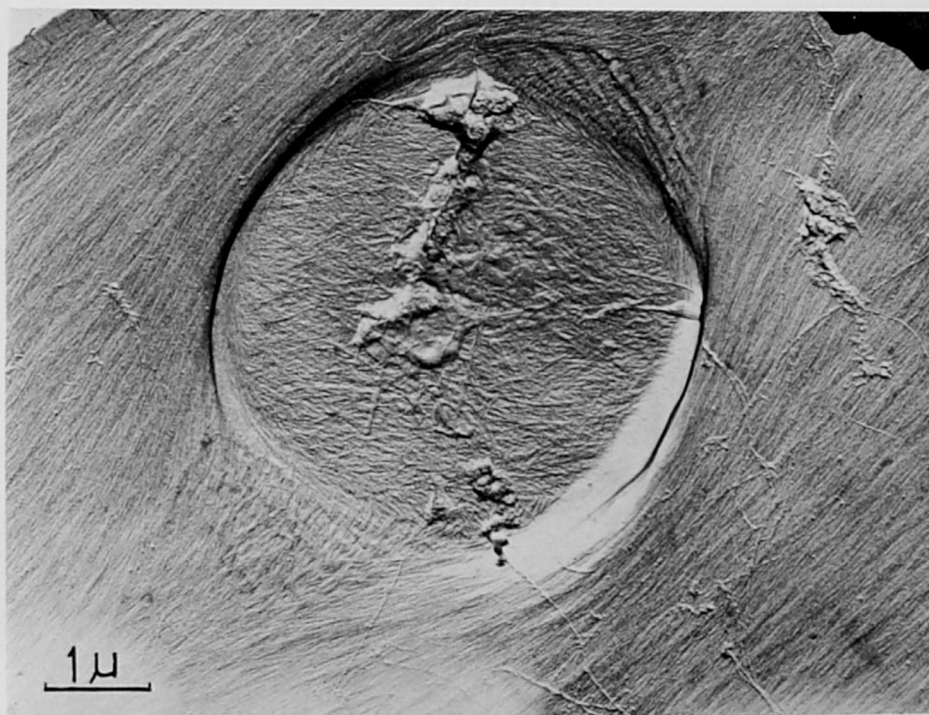


Fig. 80



Fig. 81

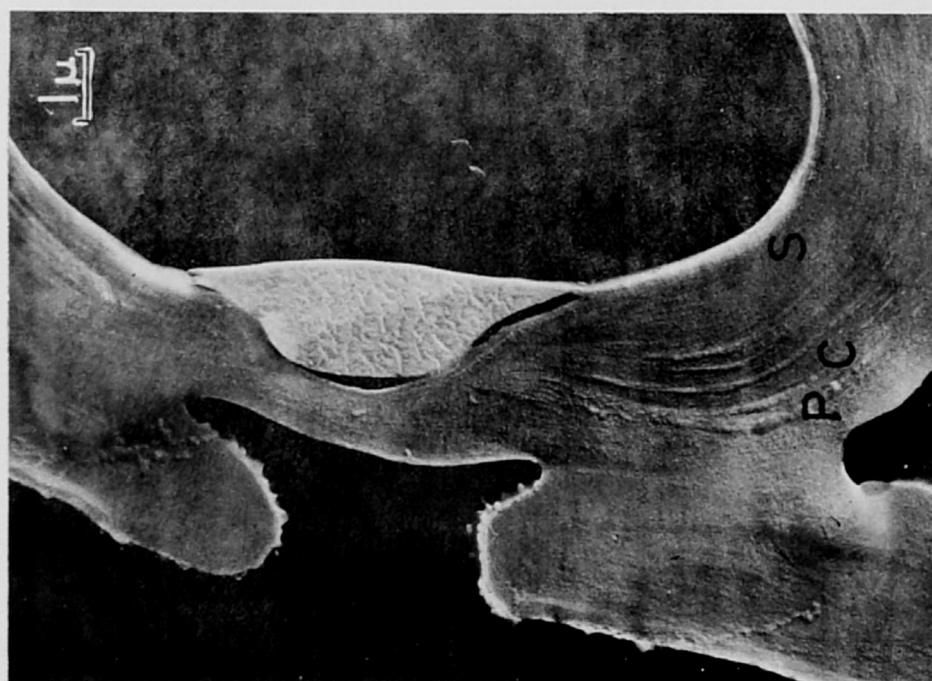


Fig. 82

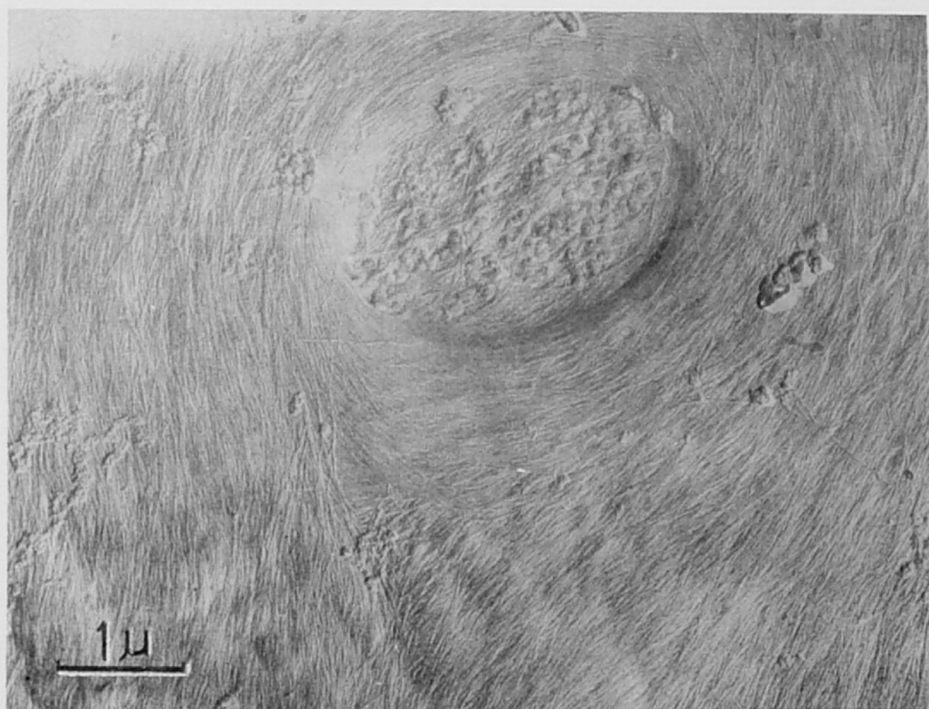


Fig. 83



Fig. 84

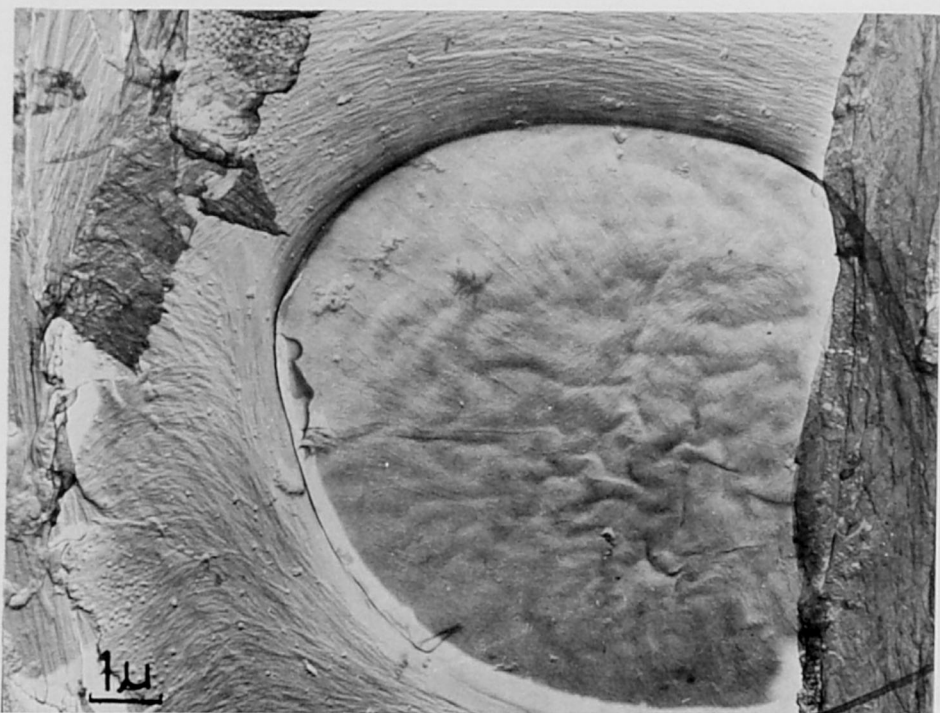


Fig. 85

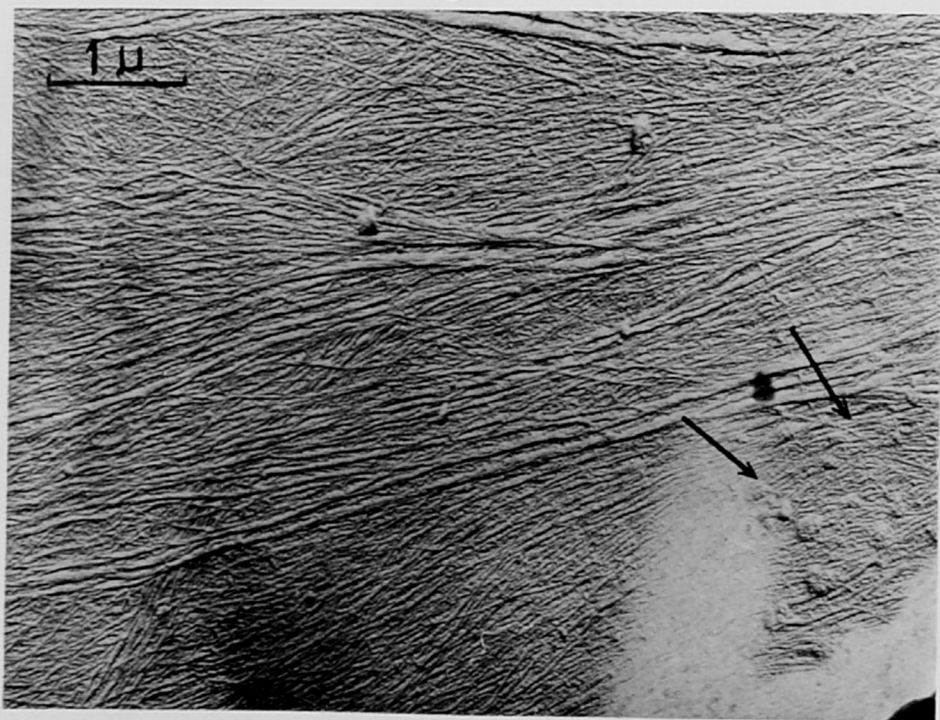


Fig. 86

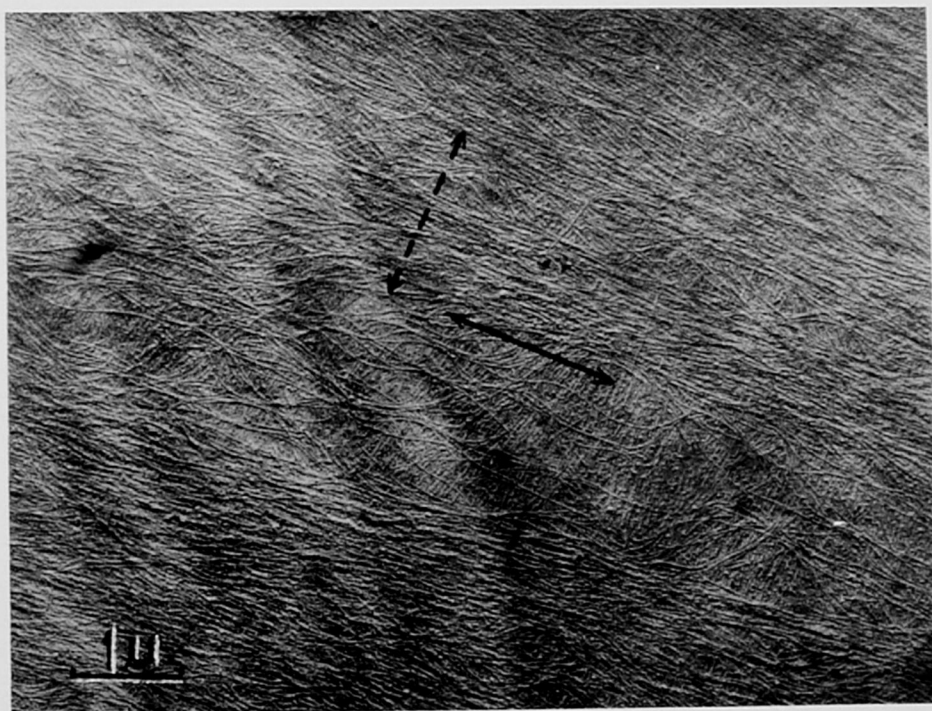


Fig. 87

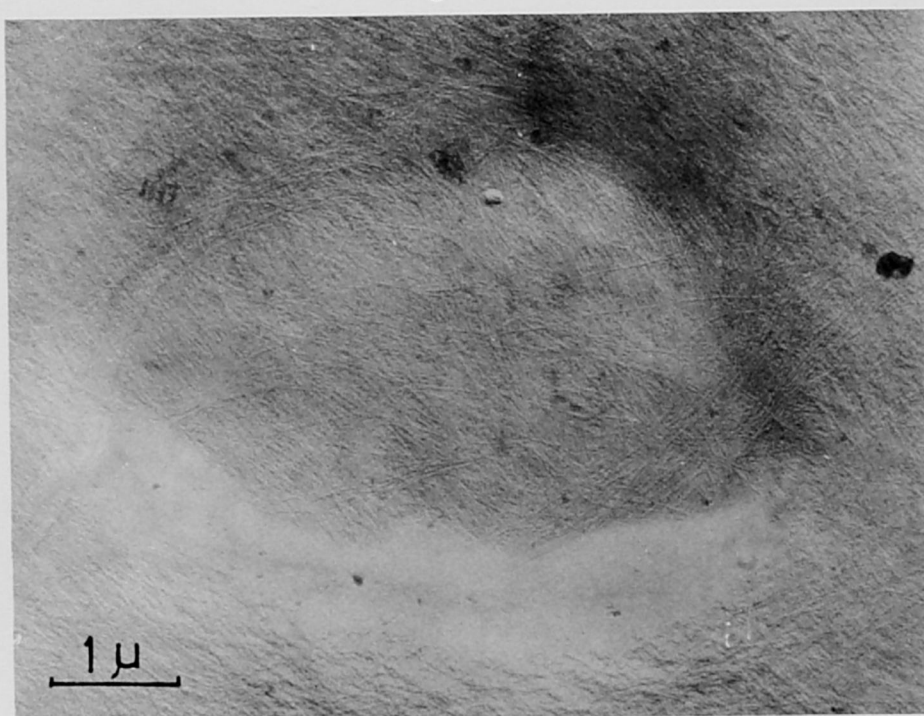


Fig. 88

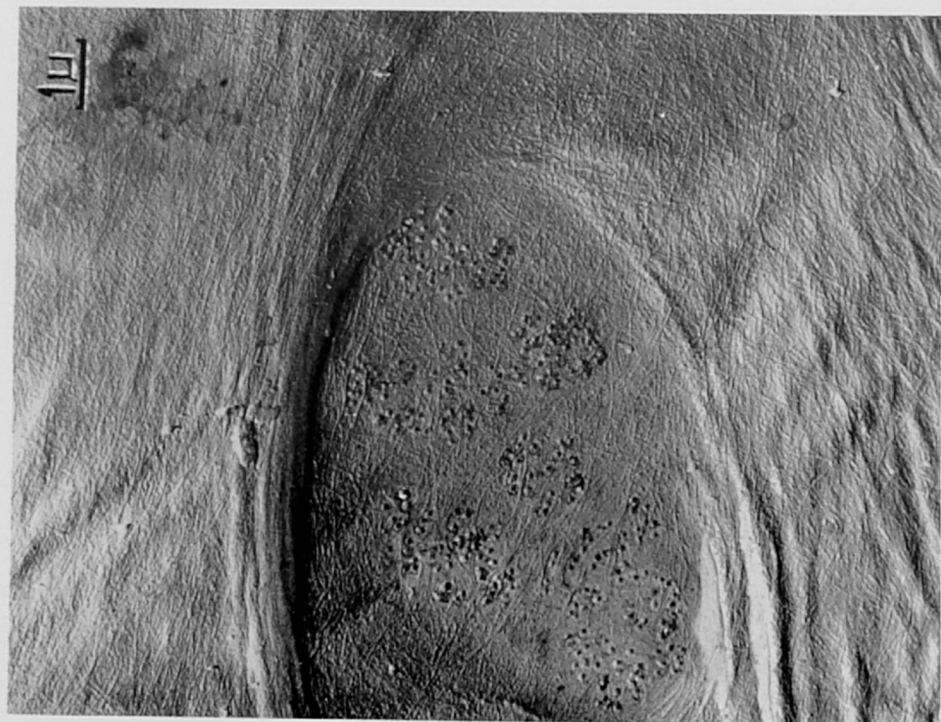


Fig. 89



Fig. 90